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Sialyl-Tn expression in bladder cancer: biological and clinical significance

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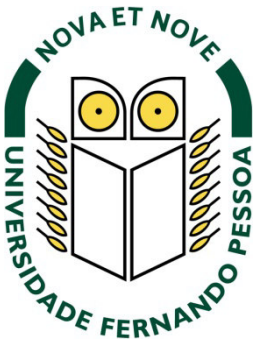
A mim…

Este trabalho foi realizado no Grupo de Patologia e Terapêutica Experimental Centro de Investigação do Instituto Português de Oncologia do Porto, e na Unidade de Investigação FP-ENAS da Universidade Fernando Pessoa.



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INDEX

RESUMO

O cancro da bexiga (CB) é considerado o segundo tumor genitourinário mais comum. Divide-se clinicopatologicamente em carcinoma urotelial da bexiga não invasor - CUBNI - menos severo e com uma baixa taxa de mortalidade e carcinoma urotelial da bexiga invasor e metastático – CUBIM - um tumor de alto grau associado a uma elevada taxa de mortalidade. Apesar de estabelecidas *guidelines* de tratamento, a mesma abordagem para tumores com o mesmo tipo histológico, nem sempre dá origem aos resultados esperados. Claramente são necessários biomarcadores objetivos que complementem a avaliação clinicopatológica convencional na previsão do prognóstico, resposta à terapêutica e na sobrevivência dos doentes com CUBNI e CUBIM.

O Sialil-Tn (STn) é um *O*-glicano resultante de uma paragem prematura no processo de glicosilação de proteínas extracelulares. A sua expressão influencia o reconhecimento por parte do sistema imunitário, afeta processos biológicos tais como o ciclo celular, apoptose, dinâmica do citoesqueleto, diminui a agregação célula-célula e aumenta a adesão extra-celular, migração, invasão e metastização tumoral. A expressão do antigénio STn tem sido descrita em diversos carcinomas humanos e em lesões preneoplásicas. Apesar desta ligação tumoral, poucos estudos têm sido publicados com o STn no CB.

O principal objetivo desta tese é melhorar a estratificação dos doentes com CUB, tendo em conta o prognóstico, através do estudo da expressão do antigénio STn. Para concretizar este objetivo, o STn é estudado em três vertentes: em relação aos aspetos biológicos do tumor, ao seu tratamento e aos aspetos clínicos da doença.

O primeiro estudo realizou-se em 69 CUB (50 CUBNI e 19 CUBI). Os resultados mostraram que STn não estava presente no urotélio histologicamente normal e a sua expressão era mais baixa nos CUBNI de baixo grau (21% tumores STn+) em comparação com os tumores de alto grau (67% tumores STn+), que incluem tumores papilares (76% tumores STn+), CIS (20% tumores STn+), e CUBI (74% tumores STn+). Em suma, estes resultados salientam uma associação entre a expressão do antigénio STn e os CUBNI de alto grau ($p<0,002$) e também com os tumores músculo-invasores ($p<0,03$). Observou-se ainda, usando um modelo

celular, que a sobreexpressão do STn aumenta a capacidade invasora das células tumorais.

Avaliou-se, ainda, a influência da expressão do STn na resposta à imunoterapia adjuvante com *Bacillus Calmette-Guérin* (BCG) em doentes com tumores com alto risco de progressão para invasão. O trabalho foi realizado numa série de 94 CUBNI (38 baixo grau, and 56 alto grau) que foram tratados com resseção transuretral seguido de imunoterapia intravesical com BCG. Aproximadamente, 66% dos tumores estudados apresentaram positividade para o STn. A expressão isolada do STn ou em combinação com o S6T (STn/S6T) associou-se com os tumores de alto grau (p=0,007; p=0,037; respetivamente), com baixas taxas de recorrência após o tratamento com BCG, e apresentavam maior sobrevivência livre de doença. Mais, as recorrências após o tratamento mostravam uma expressão reduzida do STn, sugerindo que o BCG pode ser mais eficaz nas células que expressam este antigénio. Dados *in vitro* confirmaram que a expressão de STn aumentava a adesão do BCG às células de bexiga e, consequentemente, a morte celular.

No terceiro estudo foram avaliados 96 tumores – 47 CUBNI e 49 CUBI; 16 de baixo grau e 80 de alto grau. A expressão do STn foi estatisticamente associada com o estádio histológico ≥T1-T4 (p<0,001), e com uma pior sobrevivência específica após o tratamento do tumor (p=0,024). Na mesma série, foram também avaliadas as expressões dos marcadores envolvidos na cascata do phosphatidylinositol-3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR). Os resultados mostraram que a ativação das proteínas da via do mTOR não era discriminatória em relação ao estádio histológico nem permitia a identificação dos doentes com pior prognóstico. Nos tumors invasores, os que expressavem STn e tinham a via do mTOR ativa (pAkt+ e/ou pmTOR+ e/ou pS6+), mostraram uma sobrevivência específica significativamente menor (p=0,027). Neste estudo a expressão do STn também foi avaliado numa série de ratinhos ICR submetidos ao agente carcinogénico N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) para desenvolverem carcinoma da bexiga. Descreve-se, pela primeira vez que tumores da bexiga quimicamente induzidos expressam STn, mimetizando o padrão de glicosilação em carcinomas humanos. Foi observado o efeito do sirolimus (um análogo da rapamicina) nos tumores. A administração do sirolimus foi eficaz nas células tumorais STn positivas.

Nesta tese, conseguiu compreender-se melhor o significado da expressão do antigénio STn no contexto dos tumores de bexiga. Demonstrou-se claramente que o STn está associado a este carcinoma, nomeadamente a tumores avançados, altamente proliferativos, à invasão e disseminação da doença, e a uma diminuição significativa da sobrevivência livre de doença. Também se concluiu que a presença de STn interfere de forma positiva na resposta à terapêutica destes tumores. Estas descobertas fazem do antigénio Sialil-Tn um marcador de prognóstico a ter em conta e sugerem a sua utilização em terapêutica guiada para cancro da bexiga.

ABSTRACT

Bladder cancer (BC) is the second most common genitourinary malignancy. It ranges from mild disease with a low mortality rate (classified as NMIBC - non muscle invasive bladder cancer) to extremely high-grade tumours associated with high mortality (classified as MIMBC - muscle invasive or metastatic disease). Although treatment guidelines are already defined, the same treatment for the same histological type, several times results in different outcomes. Clearly, objective biomarkers are needed in clinics to complement conventional clinicopathological evaluation to predict prognosis, treatment response and survival in both NMIBC and MIMBC patients.

Syalil-Tn (STn) is an *O*-glycan resulting of a premature stop in the glycosylation process of the extracellular proteins. Its expression influences cell recognition by the immune system, affecting processes as cell cycle, apoptosis, and actin cytoskeleton dynamics, decrease cell-cell aggregation and increase extra-cellular adhesion, migration, invasion and cancer metastization. Overexpression of STn antigen has been detected in several human carcinomas and preneoplastic lesions. Despite the cancer-associated nature of the STn antigen, few studies have been presented for BC.

The main objective of this thesis is to improve BC patient's stratification using STn expression. In order to accomplish it, STn is studied in three dimensions: relating it with tumour biology, with the tumour treatment and with the clinical outcome.

The first study used 69 urothelial BC patients (50 NMIBC and 19 MIBC). The results showed that STn was not expressed in the normal urothelium and its expression was lower in low-grade (LG) NMIBC (21% STn+ tumours) compared to high-grade lesions (HG; 67%), which include papillary tumours (76% STn+ tumours), CIS (20% of STn+ tumours), and MIBC (74% STn+ tumours). Altogether, these results highlight an association between the STn antigen and high grade NMIBC ($p<0.002$) as well as with muscle invasive tumours ($p<0.03$).

Using a cell line model, it was observed that the STn overexpression increases the invasive capacity of BC cells.

It was evaluated the STn expression role in the response to adjuvante immunotherapy with BCG (*Bacillus Calmette-Guérin*) in patients with high risk BC.The study was performed in a series of 94 (38 LG, and 56 HG) NMIBC treated

with transurethral resection followed by for BCG intravesical immunotherapy. Approximately 66% of the studied BC were STn positive. STn expression alone or in combination with S6T (STn/S6T) was associated with high-grade tumours ($p=0.007$; $p=0.037$, respectively), being also associated with lower recurrence rates after BCG, and presented longer recurrence free survival (RFS). Moreover, recurrences after treatment displayed a reduced expression of STn antigens suggesting that BCG may be more effective against cells expressing these glycans. *In vitro* data confirmed that STn expression increased BCG adhesion to bladder cells, and consequently cell death.

In the third study 96 tumours were evaluated – 47 NMIBC and 49 MIBC; 16 as LG and 80 as HG. STn expression was statistically associated with histological stage ≥T1-T4 ($p<0.001$), and with worst cancer specific survival (CSS; $p=0.024$). In the same series, the expression of phosphatidylinositol-3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) pathway markers was also addressed. In the studied series the results showed that the activation of mTOR pathway proteins did not discriminate the stage of disease and did not allow the identification of patients facing worst prognosis. In advanced stage STn expressing tumours with an active mTOR cascade (positive pAkt and/or pmTOR and/or pS6) showed a significantly worst CSS ($p=0.027$).

STn expression was also evaluated in a series of ICR mice that were submitted to the carcinogenic agent N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) to develop BC. For the first time, it is described that chemically-induced bladder tumours express the STn antigen, also mimicking the glycosylation pattern of human tumours. The effect of sirolimus in the tumours of the same mice was observed. The administration of sirolimus was effective against STn and pS6 positive cells.

In this work, a more comprehensive description of STn antigen expression in BC was achieved. It was clearly demonstrated that STn is associated with this cancer, and advanced and highly proliferative tumours, invasion and organ disseminated disease, and with a significant decrease in cancer specific survival. It also interferes positively with the response to treatment. All of these findings engender STn a prognostic marker for BC and suggest its usefulness in guided therapy for this cancer.

1. INTRODUCTION

1.1. Bladder Cancer

Bladder cancer (BC) is the second most common genitourinary malignancy [1]. It is the fourth most prevalent disease in the United States accounting for 7 percent of cancers in men [2]. Bladder cancer ranges from mild disease with a low mortality rate to extremely high-grade tumours associated with high mortality [3]. Accurate staging and grading is important to select optimal treatment. Bladder cancer has a clear correlation with environmental exposures, such as smoking [3]. High recurrence rates, long-term follow-up, and the frequent need for repeated interventions are the major causes that turn BC the costliest to treat among all solid tumours [3,4,5]. Therefore, accurate staging and grading are essential to the refinement of the prognosis and better allocation of treatment resources.

Epidemiology

An estimated 386,300 new cases and 150,200 deaths from BC occurred in 2008 worldwide. The majority of BC occurs in males and there is a 14-fold variation in incidence internationally. The highest incidence rates are found in the countries of Europe, North America, and Northern Africa. Egyptian males have the highest mortality rates (16.3 per 100,000), which is twice as high as the highest rates in Europe (8.3 in Spain and 8.0 in Poland) and over 4 times higher than that in the United States (3.7). The lowest rates are found in the countries of Melanesia and Middle Africa [6].

Bladder cancer is the 7th most common cancer in men and the 17th most common in women worldwide. BC is more common in developed countries being the 4th and 9th most common cancer in men and women, respectively, in the Western world [6]. In the United States, in 2007, approximately 50,000 men and 17,000 women were diagnosed with BC, and near 14,000 succumbed by this disease [1]. About 74,690 new cases were expected for 2014, being 56,390 in men, with an estimated death of 11,170 men from this cancer [2]. BC is primarily found in elder persons, with approximately 80 percent of new cases occurring around 60 years or older [7]. BC is about three times more common in men (one in 27) than women

(one in 85). It is more prevalent in white persons; however, because of delayed diagnosis, mortality rates are higher in black persons [8].
According to the *Registos Oncológicos Regionais*, in Portugal, in 2006 were diagnosed 1711 new BC, 1336 of which affecting men and 375 affecting women. In the same year, 514 men and 186 women died from this malignancy turning BC the 7th most prevalent type of tumor and the 8th most common cause of death from cancer [9].

Risk factors

Well established risk factors for bladder cancer include tobacco use, infections with *Schistosoma haematobium*, and occupational exposures to aromatic amines and polycyclic aromatic hydrocarbons [10,11]. Other suspected risk factors for BC include dietary patterns, environmental pollution, and genetic predisposition [11]. Age is also considered a bladder cancer risk factor because it occurs predominantly in the elder [3].
Incidence and mortality rates vary globally, mainly due to differences in risk factors. While smoking is the predominant cause of bladder cancer in the United States, infection with *S. haematobium* is the major cause of the disease in most parts of Africa [12].
According to Burger *et al.* (2013) the understanding of genetic risks in BC is growing steadily [11]. The risk for this malignancy is two-fold higher in first-degree relatives of BC patients. Inherited genetic factors, such as the genetic slow acetylator N-acetyltransferase 2 (NAT2) variants and glutathione S-transferase mu 1 (GSTM1)–null genotypes, have been established as risk factors for BC. Factors such as slow acetylation may not intrinsically lead to BC but may confer additional risk to exposure of carcinogens such as tobacco products [11].
Smoking is the most important risk factor for BC, having a direct pathophysiologic link between them [11]. Smokers are at least 3 times as likely to develop BC as nonsmokers. Smoking causes about half of the BC in both men and women [13]. Tobacco smoke contains aromatic amines, such as b-naphthylamine, and polycyclic aromatic hydrocarbons known to cause BC. Moreover, these compounds are renally excreted and exert a carcinogenic effect on the entire urinary system. Tobacco consumption is common, and thus its epidemiologic impact is massive [11].

Following smoking, occupational exposure to carcinogens is viewed as the second most relevant risk factor for BC [11,14]. In industrialized countries, 5 to 10 percent of diagnosed BC are caused by occupational exposures to aromatic amines (benzidine, 4-aminobiphenyl, 2-naphthylamine, 4-chloro-o-toluidine) used in the manufacturing of chemical dyes, pharmaceuticals and in gas treatment plants [11,14]. Some studies show that sometimes the disease appears only 30 to 50 years after exposure [3].

Bladder exposure to radiation, often as treatment for other pelvic malignancies, as well as cyclophosfamide and other similar chemotherapeutic agents, has been linked to secondary BC, which may be particularly aggressive. These cancers tend to be high grade and locally advanced [15,16].

Clinical presentation

Painless visible hematuria is the most common presenting symptom in patients diagnosed with BC [16,17]. However, symptoms of bladder irritation, such as urinary frequency and urgency, more commonly occur in patients with bladder carcinoma *in situ* (CIS). Obstructive symptoms may be present if the tumour is located near the urethra or bladder neck. In advanced disease, patients may present flank pain caused by uretheral obstruction, or with abdominal, pelvic, or bone pain from distant metastasis. Early bladder cancer is not detectable by physical examination, however, a palpable kidney or pelvic mass may be present in advanced and metastatic disease (Table 1) [17,18].

Table 1. Signs and Symptoms of Bladder Cancer (adapted from [3])

Hematuria (gross or microscopic)
Irritative symptoms
Dysuria, frequency, urge incontinence, urgency
Obstructive symptoms
Decreased force of stream, feeling of incomplete voiding, intermittent stream, straining
Signs and symptoms of metastases or advanced disease
Abdominal, bone, flank, or pelvic pain; anorexia, cachexia, or pallor; lower extremity edema; renal failure; respiratory symptoms (e.g., cough, dyspnea, hemoptysis); suprapubic palpable mass

Clinical and biological evolution

Approximately 90 percent of bladder tumours are urothelial carcinomas (before known as transitional cell carcinomas); the remaining 10 percent are nonurothelial or mesenchymal in origin [16,17].

Seventy to eighty percent of newly diagnosed bladder tumours are non-muscle invasive bladder tumours (NMIBC), staging from Tis to Ta and T1 (Figure 1). At diagnosis, the remaining tumours correspond to muscle invasive bladder cancer (MIBC), staging from T2 to T4, or metastatic disease (Figure 1) [15,19].

NMIBC, previously known as superficial bladder cancer, is defined as a tumour that is confined to the first two layers of urinary bladder tissue, the urothelium (Ta) and the *lamina propria* (T1). A superficial tumour that projects toward the bladder lumen is considered papillary. In turn, a flat carcinoma *in situ* is confined to the urothelium and does not project outward, but carries a higher risk of progressing and invading inward. Once the tumour has progressed to the smooth muscle and beyond, it is considered an invasive bladder tumour (T2-T4) (Figure 1) [20].

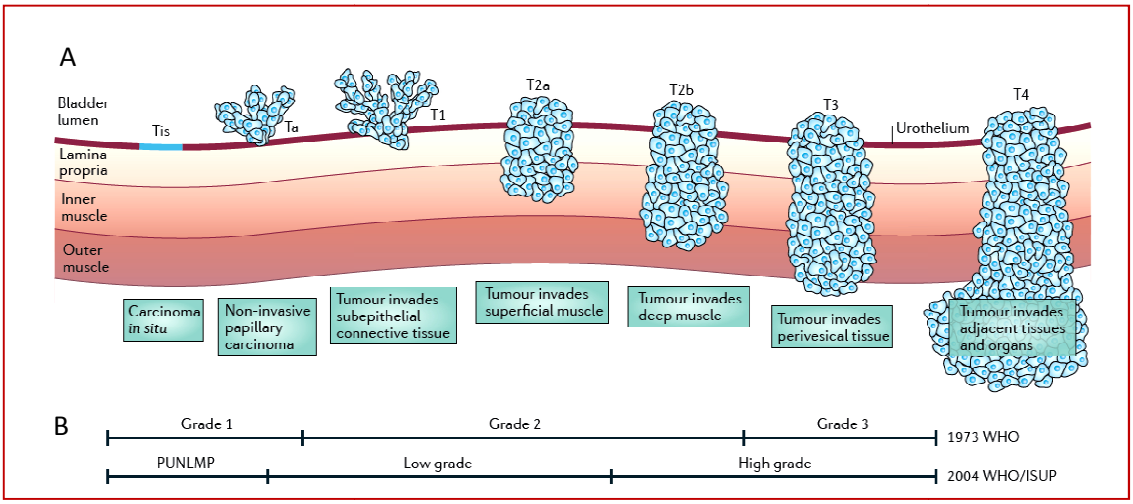

Figure 1 – Representation of bladder cancer grading and staging. a) Staging of bladder cancer according to the Tumor-Node-Metastasis (TNM) system. b) Grading before and after 1998, according to the WHO/ISUP criteria. Note that the major difference is the classification grade 1 tumours, now denominated papillary urothelial neoplasm of low malignant potential (PUNLMP). Adapted from [20].

Fifty to seventy percent of NMIBC tend to recur within 5 years after surgical excision, rarely progressing to invasive cancer (10-30%). The vast majority of MIBC occur in patients without a prior history of papillary tumours and have a poor prognosis [3,21].

In 1998, a new histological grading classification of non-invasive urothelial tumours was proposed by the World Health Organization (WHO) and the International Society of Urological Pathology (ISUP) (1998 WHO/ISUP classification) and published by the WHO in 2004 [22]. According to this new classification, grade 1 tumors are reclassified has papillary urothelial neoplasms of low malignant potential (PUNLMP). Although these lesions do not have cytological features of malignancy, they show normal urothelial cells in a papillary configuration. In spite of PUNLMP have a negligible risk for progression, they are not completely benign and still have a tendency to recur. In addition, the intermediate grade (Grade 2) has been eliminated in this new classification (Figure 1; Table 2).

Table 2: WHO grading in 1973 and in 2004 (adapted from [22])

1973 WHO grading
Urothelial papiloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
2004 WHO grading
Urothelial papilloma (completely benign lesion)
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

Based on available prognostic factors and particularly data from the European Organization for Research and Treatment of Cancer (EORTC) risk tables, the Guidelines Panel recommends stratification of patients with non-muscle invasive bladder cancer into three risk groups, such as low-risk, intermediate-risk and high-risk (Table 3). This group stratification enables the prediction of the risk of

recurrence and progression based on the clinicopathological characteristics of the tumour [22].

Table 3: Risk group stratification (adapted from [22])

Low-risk tumours
Primary, solitary, Ta, G1 (low grade), < 3 cm, no CIS
Intermediate-risk tumours
All tumours not defined in the two adjacent categories (between the category of low and high risk)
High-risk tumours; any of the following;
T1 tumour G3 (high grade) tumour CIS Multiple and recurrent and large (> 3 cm) Ta G1G2 (low grade) tumours (all conditions must be presented in this point)

The tumour, node, metastasis (TNM) classification of malignant tumours is the method most widely used to classify the extent of cancer spread (Figure 2) [23].

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma in situ: "flat tumor"
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscularis propria
pT2a	Tumor invades superficial muscularis propria (inner half)
pT2b	Tumor invades deep muscularis propria (outer half)
T3	Tumor invades perivesical tissue
pT3a	Microscopically
pT3b	Macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostatic stroma, uterus, vagina
T4b	Tumor invades pelvic wall, abdominal wall

Regional Lymph Nodes (N)

Regional lymph nodes include both primary and secondary drainage regions. All other nodes above the aortic bifurcation are considered distant lymph nodes.

NX	Lymph nodes cannot be assessed
N0	No lymph node metastasis
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node)
N2	Mutiple regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node metastasis)
N3	Lymph node metastasis to the common iliac lymph nodes

Distant Metastasis (M)

M0	No distant metastasis
M1	Distant metastasis

Figure 2- **T**umor, **N**ode and **M**etastasis (TNM) tumor classification. Clinical classification of bladder tumour according to its invasive potential, nodal *status* and the presence of distance metastasis. Reprinted from [3].

The TNM staging system provides valuable information that helps predict outcomes and direct clinical resources. Progressive stages are associated with worse 5-year survival rates [14]. Given all the variables not accounted in the TNM staging system, there is a need for a more accurate patient stratification. A more precise stratification would allow patients with high risk of recurrence to be treated more aggressively, while sparing low risk patients from overtreatment [3].
Most BC prognostic factors rely on the histopathological classification of the tumour, as well as on the tumor size, stage and grade [15]. Moreover, blood vessel invasion, multifocality, presence of concomitant CIS, nodal *status* and the existence of distance metastasis are important BC prognostic factors as well [15].

Figure 3 resumes the clinical evolution of urothelial bladder cancer, according to Jaccobs *et al* (2010) [3].

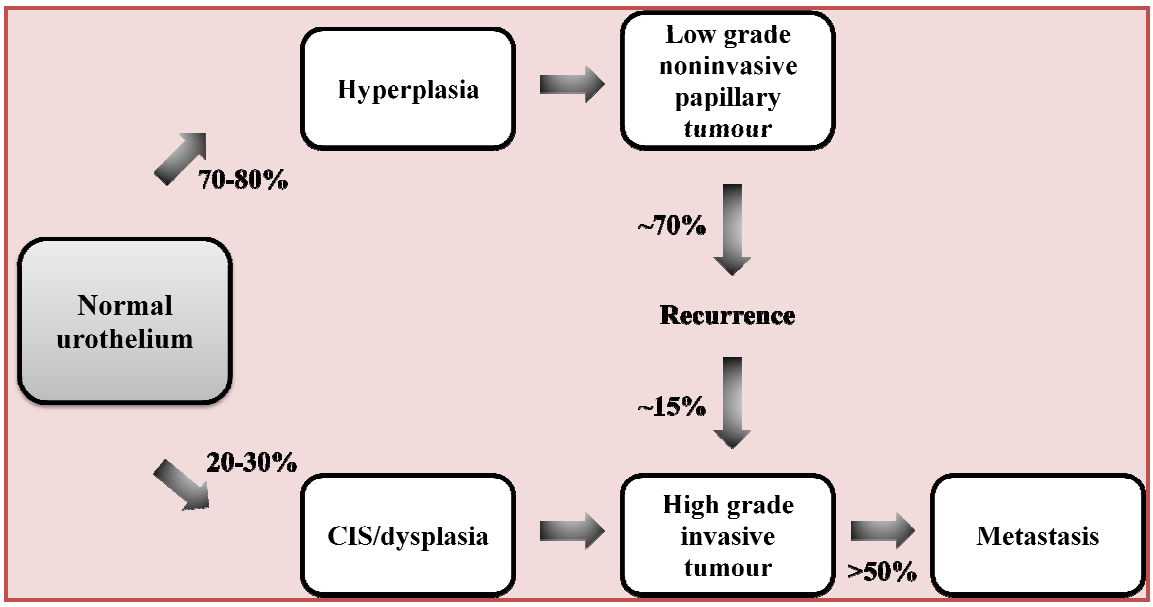

Figure 3 – Urothelial cancer origin and progression. Approximately 70% to 80% of urothelial cancers are papillary lesions, which arise from hyperplasic epithelium with the expansion of a preneoplasic clone, showing minimal phenotypic deviation from normal urothelium. These lesions tend to become low grade and noninvasive tumors. About 70% of these cases recur, with 15% of which progressing to high grade invasive tumors. Nonpapillary and invasive tumours usually arise from severe dysplasia or carcinoma *in situ* (CIS) and over 50% of these cases progress to metastatic disease. Adapted from [3].

Diagnosis

Clinical investigation should begin with the careful knowledge of patient's background, including any associated risk factors. Patients with urinary symptoms should have a urinalysis with urine microscopy and a urine culture to rule out infection [24].

The primary means of BC diagnosis and surveillance are the cystoscopic examination of the bladder, as well as the cytologic examination of the cellular material present in the urine [24].
Urine cytology has a high specificity (95 to 100 percent), but a low sensitivity (66 to 79 percent) for the detection of BC. Urine cytology is frequently used to identify high-grade tumours and monitor patients for persistent or recurrent disease following treatment. Moreover, bladder wash cytology detects *in situ* carcinoma in almost all cases [16]. However, it fails to detect BC in asymptomatic patients [16].

A number of noninvasive tests to detect BC have been developed and approved for clinical practice by the Food and Drug Administration agency (FDA), namely, BTA STAT, BTA TRAK, NMP-22, ImmunoCyt/uCytt and UroVysion [25]. It is interesting to note that almost all tumour markers have a much better sensitivity than cytology but few reached the same level of specificity. Nevertheless, cystoscopy remains the mainstay of diagnosis and surveillance, providing information about the tumour location, appearance, and size. Detection of flat neoplastic lesions, such as *in situ* carcinoma, can be improved by using fluorescence cystoscopy [16].
Other means of diagnosis include bladder biopsies, computerized tomography or magnetic resonance imaging scans, which is particularly useful to evaluate the presence of bladder wall thickening, perivesical fat invasion, and lymph node involvement [23]. In turn, transurethral resection of the tumour (TURBT) is indicated when the tumour is localized and cytologies are suggestive of malignancy [15].

Treatment

Optimal treatment involves a multidisciplinary approach, combining the urology, pathology, and oncology fields. After the diagnosis, several treatments can be implemented according with the grade and stage of the tumour. Moreover, a close follow-up is essential for BC patients [16].

- Nonmuscle invasive bladder cancer

TURBT is one of the most common procedures used by practicing urologists for the diagnosis, staging and treatment of NMIBC [26].

The standard treatment of Ta and T1 BC is complete endoscopic resection, with or without adjuvant intravesical therapy [15]. Concomitant intravesical therapy depends on the depth and grade of tumour invasion, completeness of resection, and estimated probability of recurrence. For instance, low-grade Ta tumors are treated with resection alone [16]. *Bacillus Calmette-Guérin* (BCG), an attenuated strain of *Mycobacterium bovis*, is the most commonly used form of intravesical immunotherapy. BCG is currently used for CIS and recurrent nonmuscle invasive disease [15]. The response to intravesical treatment with BCG or chemotherapy is an important prognostic factor for subsequent progression and death caused by BC. Approximately 10-20% of complete responders eventually progress to muscle-invasive disease, compared with 66% of non-responders [22].

Mitomycin C, epirubicin, and doxorubicin are also used as adjuvant therapy and have all shown a beneficial effect, with no efficacy comparisons made between the drugs [22]. Although BCG therapy causes significantly more side effects than chemotherapy, it appears to be significantly better at preventing recurrences than regimens based on mitomycin C (MMC) or epirubicin [22].

- Muscle invasive bladder cancer

Radical cystectomy with pelvic lymphadenectomy is the standard treatment for MIBC (stage ≥T2) [16,23]. However, only 50% of patients exceed the 5-year survival mark when submitted to this procedure. The use of peri-operative chemotherapy has been explored since the 1980s. There are many advantages of administering chemotherapy before surgery or radiation therapy, such as reduction

of tumor size and possible reduction of micrometastatic disease [23]. According to BC guidelines 2013, neoadjuvant cisplatinum-based combination therapy is recommended for T2-T4a, and cN0M0 BC [23]. The combination therapy based on gemcitabine (Gemzar) and cisplatin (Platinol) is the standard adjuvant treatment for most patients because of its lower toxicity, when compared to other combinations, such as the MVAC regimen (methotrexate, vinblastine, doxorubicin – Adriamycin – and cisplatin) [16]. Table 4 resumes the standard treatments of urothelial bladder carcinoma according to its clinical features.

Table 4. Treatment of Urothelial Bladder Carcinoma (Adapted from [16])

Tumour	***Treatment***
Low-grade Ta	Transurethral resection without intravesical chemotherapy Some experts suggest a single dose of intravesical chemotherapy (not immunotherapy) within 24 hours of resection to prevent recurrence
High-grade Ta	Repeat transurethral resection (if lymphovascular invasion, incomplete resection, or no muscle in the specimen), consider intravesical BCG (preferred) or mitomycin
Carcinoma in situ/ tumour in situ	Transurethral resection followed by intravesical BCG once a week for six weeks
Low-grade T1	Repeat transurethral resection followed by intravesical BCG (preferred) or mitomycin
High-grade T1	Repeat transurethral resection, followed by intravesical BCG or mitomycin, or cystectomy
T2a or T2b (organ confined)	Radical cystectomy followed by chemotherapy in high-risk patients (e.g., those with nodal involvement, highgrade histology, transmural or vascular invasion, pathologic T3 lesion)
T3a or T3b	Radical cystectomy followed by adjuvant chemotherapy, consider neoadjuvant Chemotherapy Two trials have shown survival benefit with neoadjuvant chemotherapy (three cycles of methotrexate, vinblastine, doxorubicin [Adriamycin], and cisplatin [Platinol]) in T2 or T3 disease
T4a, T4b, or metastatic disease	Chemotherapy alone or in combination with radiation therapy, except in high-risk patients (e.g., those with poor performance status, visceral [lung or liver] disease, bone disease, poor cardiac status) Radiation therapy not routinely used in the United States for locally advanced bladder cancer

BCG = bacille Calmette-Guérin.

As previously described, muscle invasive bladder cancer is amongst the most common and deadliest genitourinary cancers. The mainstay treatment is cisplatin-

based regimens, which fail in avoiding tumour relapse and disease dissemination, urging novel biomarkers for accurate patient stratification and new therapeutics.

1.2. Glycosylation and Sialyl-Tn

Protein *O*-glycosylation in eukaryotic cells

Complex carbohydrates are the *major* components of the cell membrane, having important biological implications in cell-cell or cell-extracelular matrix interactions, as well as signal transduction. There is a distinct difference in carbohydrate profiles between normal and tumour tissues. Aberrant glycosilation is responsible for the characteristic carbohydrate expression associated with malignant transformation, being catalysed by specific glycosyltransferases and glycosidases [27]. Glycosylation is the most common post-translational modification in proteins and plays a pivotal role in structural and functional features of several molecules – in fact, virtually, all proteins can be glycosylated [28,29]. It mainly affects the outer part of the carbohydrate moiety of glycoproteins and glycolipids, leading to the expression of tumour-associated carbohydrate antigens (TACAs). Many TACAs are sialylated structures and the general increase in cell surface glycoproteins sialylation is commonly observed in *N-linked* and *O*-linked oligosaccharides of carcinoma cells [30]. Altered or aberrant glycosylation is common in several human carcinomas, especially during tumour progression, modifying cellular adhesion and motility, affecting their invasive and metastatic potential [31].
Protein *O*-glycosylation is a stepwise pathway that begins in the Golgi apparatus of eukaryotic cells, in which monosaccharides are added individually and sequentially to an hydroxyl oxygen in Serine/Threonine (Ser/Thr) tandem repeat regions of proteins by a complex set of enzymes (Figure 4) [29,32].

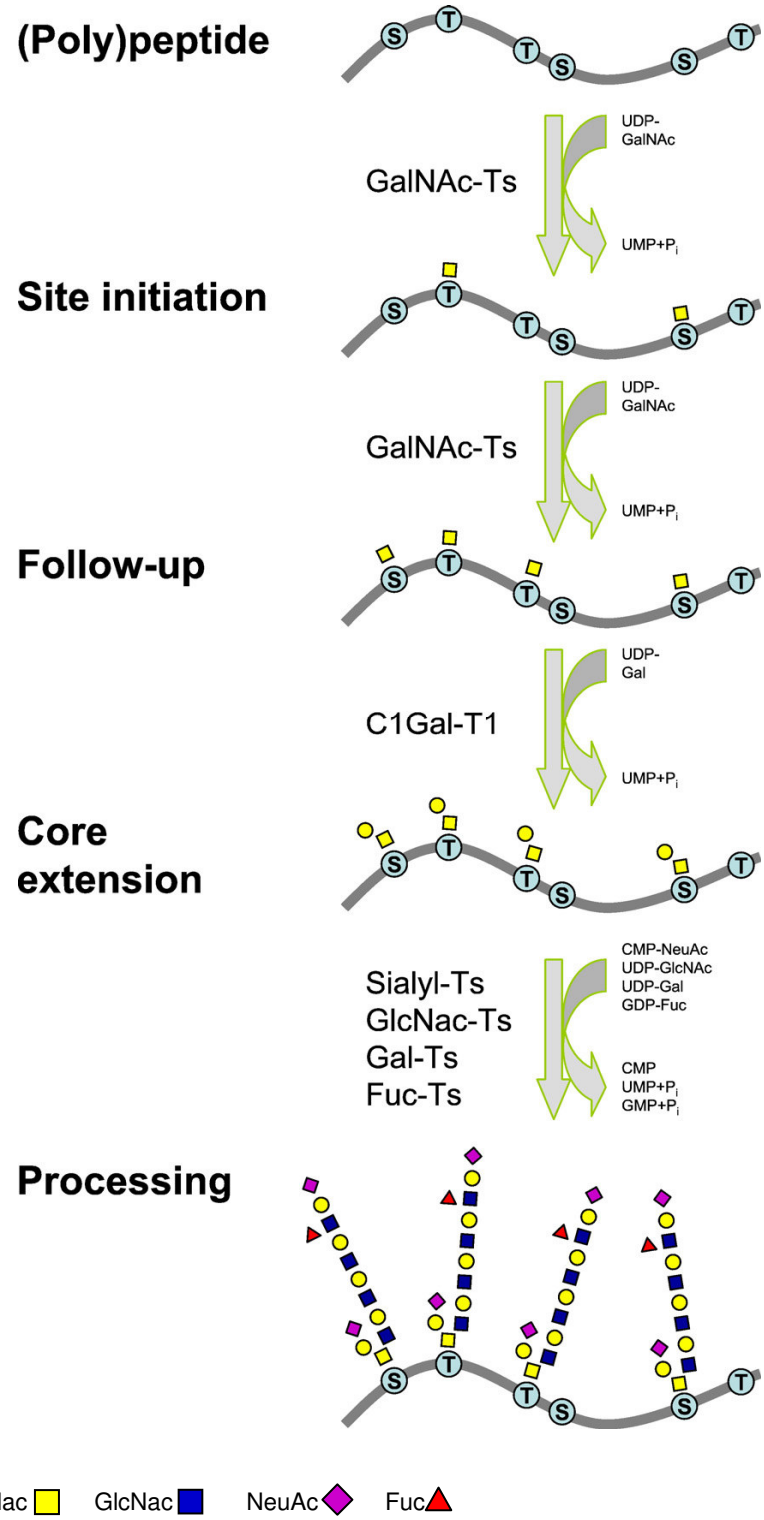

Figure 4 - Steps of mucin-type *O*-glycan biosynthesis. O-glycosylation occurs post-translationally by covalently α-linking a GalNAc moiety from a sugar donor UDP-GalNAc to protein serine (S) or threonine (T) residues by UDPGalNAc-polypeptide N-acetylgalactosaminyl- transferases (ppGalNAc-Ts). After the first glycan (GalNAc) is added, forming the Tn antigen, the core 1 structure is synthesised by the Gal-transferase (β(1-3)-galactosyltransferase, C1Gal-T1 or T-synthase), which adds Gal to GalNAc. Core 1 may function as a precursor of other core structures (from core 2 to 8), by the addition of different monosaccharides, such as galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine and sialic acids. Adapted from [45].

O-glycans are highly expressed in mucins, a class of heavily glycosylated proteins secreted by mucosa and some exocrine glands [28,33]. These high molecular weight proteins are rich in Ser and Thr residues, providing the necessary template for *O*-glycosylation [28,33-35]. As a result of its association with mucins, cell surface *O*-glycans are commonly designated as mucin-type *O*-glycans; nevertheless, *O*-glycans can also be found in many other cell glycoproteins exhibiting Ser and Thr residues [28]. Nuclear *O*-glycosylation can also occur in the cells and it is a process in which a certain variation of *O*-glycosylation is seen in the nucleus and cytosol. This *O*-glycosylation is characterized by a single *N*-acetylglucosamine (GlcNAc) attachment to Ser/Thr residues, and appears to have a signaling role similar to protein phosphorylation [36,37]. Mucin-type *O*-glycans synthesis begins with the transfer of an α-*N*-acetylgalactosamine (GalNAc) from the donor-nucleotide sugar uridine diphosphate – *N*-acetylgalactosamine (UDP-GalNAc) to the hydroxyl group of a residue of Ser/Thr within the glycoprotein being synthesized [28,29,38]. This reaction forms the simplest mucin *O*-glycan and the only structure common to all *O*-glycans – the Tn antigen (GalNAcα-*O*-Ser/Thr) [35]. This antigen can be sialylated at the *O*-6 position, generating the sialyl-Tn (STn, Neu5Acα2-6GalNAcα-*O*-Ser/Thr) antigen, by the action of the ST6GalNAc-I sialyltransferase. The sialylation of this *O*-glycan stops further chain elongation (Figure 5) [28,39,40]. Alternatively, the Tn antigen can give raise to other core structures [35].

Carbohydrate structures formed by a GalNAc or by a Galβ1-3GalNac disaccharide O-linked to Ser/Thr, as well as their sialylated forms are collectively denominated as Thomsen-Friedenreich-related antigens (TF-antigens) (Figure 5). As previously described, their biosynthesis depends on a coordinated action of several glycosyltransferases [40].

The initial step of *O*-glycosylation is catalyzed by a family of 20 membrane-bound enzymes denominated UDP-GalNAc:polypeptide glycosyltransferases (ppGalNAc-Ts), that have distinct but overlapping specificities [29,32]. This family of enzymes has a C-terminal lectin domain, which makes them unique among all others eukaryotic glycosyltransferases [36,41]. This diversity, as well as the fact that *O*-glycosylation sequence consensus motif has not emerged yet, allows a fine tuned

control of the initiation of this process in a specific cell or even in a specific protein [28,42].

Core 1 structure, also called T antigen or Thomsen-Friedenreich antigen (Galβ1-3GalNAcα-*O*-Ser/Thr) is the result of the addiction of a galactose (Gal) residue, transferred from an uridine diphosphate - galactosamine (UDP-Gal) donor, to the GalNAc of the Tn antigen by a specific galactosyltransferase (β(1-3)-galactosyltransferase, Core1Gal-T1 or T-synthase), as shown in Figure 5 [28,40,42].

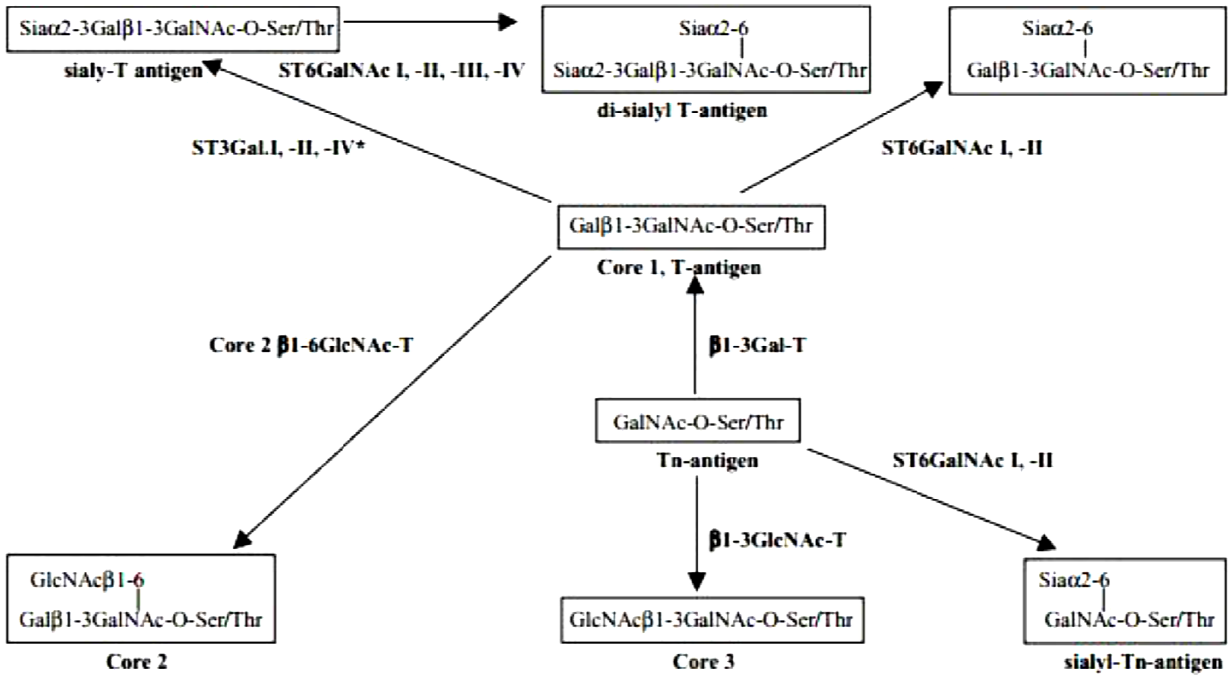

Figure 5 - Schematic representation of the biosynthesis of Thomsen-Friedenreich-related antigens. The Tn antigen is originated by the addition of a GalNAc to serine or threonine residues of the polypeptide chain, it can be transformed in sialyl-Tn antigen by the action of ST6GalNAc I or -II, or can be elongated by the addition of a *β*1-3-linked galactose, becoming the core 1 structure (T-antigen) or by the addition of a *β*1-3GlcNAc, yielding the core 3 structure. The T antigen can be elongated by the addition of a GlcNAc *β*1-6-linked to GalNAc, generating the core 2 structure; if a sialic acid is additioned in *α*2-3-linkage to Gal by ST3Gal I or ST3Gal II or ST3Gal IV, core 2 becomes the sialyl-T antigen which, in turns, can be further sialylated by ST6GalNAc I, -II, -III or -IV, generating the di-sialyl T-antigen. Core 1 structure can be also directly sialylated on the GalNAc residue by ST6GalNAc I or II, yielding the S6T antigen. Adapted from [40].

Ju *et al.* (2002) and later, Wang *et al.* (2009) proposed that the expression of T-synthase is under control of the co-expression of an unique molecular chaperone localized in the endoplasmatic reticulum (ER), Cosmc. Human *Cosmc* resides on Xq24 as a single exon gene and, apparently, this chaperone prevents the

aggregation and proteosomal degradation of T-synthase, and is required for the export of the enzyme from the ER, leading to the formation of core 1 [43,44].

T antigen can be sialylated at *O*-3 position by a ST3Gal-sialyltransferase, yielding the sialyl-3-T antigen (S3T, Neu5Acα2-3Galβ1-3GalNAcα-*O*-Ser/Thr); this antigen can be further sialylated at *O*-6 position, by a ST6Gal-sialyltransferase, originating the di-sialyl-T antigen (diST, Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-*O*-Ser/Thr) (Figure 5) [28,40]. Alternatively, the GalNAc residue of the T antigen may be sialylated at the *O*-6 position by a ST6GalNAc-sialyltransferase, yielding the sialyl-6-T antigen (S6T, Galβ1-3(Neu5Acα2-6) GalNAcα-*O*-Ser/Thr), which can then originate the diST antigen (Figure 5). Tn and T antigens, as well as their sialylated structures that block further elongation of the *O*-chain, are generally designated simple mucin-type *O*-glycans [33,35].

Core 1 functions as a precursor of other core structures (from core 2 to 8), by the addition of different monosaccharides, such as galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acids [32,36].

Core 2 is synthesized in many epithelial (as intestinal mucosa) and hematopoietic tissues by the addition of a branching GlcNAcβ(1-6)-linked residue to core 1, by core 2 β(1-6) *N*-acetylglucosaminyltransferases (or C2Gn-T) (Figure 5) [28,40,45].

Alternatively to core 1 *O*-glycan formation, GlcNAc instead of Gal can be transferred in a β1–3 linkage by a core 3 β(1-3) *N*-acetylglucosaminetransferase (C3Gn-T3) to Tn antigen forming the core 3 structure, as represented in Figure 5, and the subsequent addition of a GlcNAc residue onto core 3 by a C2Gn-T yields core 4. Thus, the prior synthesis of core 3 is required for the formation of core 4 [32,40,45].

The extension of core units provides a vast array of glycan structures, and is catalyzed by *N*-β3/4-acetylglucosaminyltransferases (β3/4 Gn-Ts) and/or β3/4-galactosyltransferases (β3/4 Gal-Ts), leading to the formation of side chains designated type-1 (Galβ1-3GlcNAc-R) and type-2 (Galβ1-4GlcNAc-R) chains [28,32,36]. These chains present a ubiquitous expression, and therefore are widely expressed among epithelial tissues. Type-1 and type-2 chains can be modified by the action of fucosyl and sialyltransferases, yielding ABO blood group determinants and/or Lewis blood group related antigens (Lea, SLea, Lex, SLex, Leb and Ley), which function as terminal structures by stopping chain elongation [28,32,36]. After *O*-glycosylation is concluded, the formed structures can undergo

modifications, such as phosphorylation, sulfation and methylation, which constitute an additional mean for their diversity [36].

O-GalNAc glycans are crucial structures for cells viability, because they play several and distinct roles in the organism, depending on the structure on which are present – this structural variability allows them to function as signaling, recognition and adhesion molecules [33,38,46].

Regulation of *O*-glycosylation

As *O*-GalNAc glycans are complex and widely diverse structures, their synthesis is tightly regulated. However, the exact mechanisms by which this regulation occurs remains to be clarified [33,34]. The regulation of *O*-glycosylation seems to be dependent on the pattern of the enzymes involved in this process – substrate specificity, intracellular localization and level of relative activity are the main control factors of *O*-glycosylation [33,34,37].

Substrate specificity of glycosyltransferases and other enzymes involved in *O*-glycosylation restricts the number of possible *O*-GalNAc glycans that can be formed, since these enzymes act in well-defined substrates [33,41]. On the other hand, this specificity reduces the number of pathways by which *O*-glycans can be synthesized [33,41]. For example, ST antigen can only be generated by the addition of a sialic acid residue to T antigen (core 1). The adding of a galactose residue to STn antigen does not yield the same structure, that if added to Tn, since the sialic acid present in STn antigen blocks the action of other glycosyltransferases, namely C1Gal-T1 [33,40].

Intracellular localization of glycosyltransferases is another important regulation factor of *O*-glycosylation, which is determined by the physical separation of enzymes within the Golgi compartments [41,35]. These enzymes appear to be arranged in an assembly line in the Golgi apparatus – early acting glycosyltransferases occupy the *cis*-Golgi, intermediate acting enzymes are localized in the medial Golgi and terminal acting enzymes in the *trans*-Golgi, which allows a tight control of *O*-glycosylation [33,35].

The third major control factor is the level of enzymatic activity of glycosyltransferases, that consequently dictate the relative amounts of

synthesized *O*-glycans. For instance, the activity of two competitive enzymes will determine the nature of the *O*-GalNAc glycan being synthesized, depending on which activity predominates [41,46]. Besides the competition between glycosyltransferases, other regulatory components influence glycosyltransferases activation, namely specific binding proteins such as the Cosmc chaperone [33,43].

Alterations of *O*-glycosylation in cancer

Aberrant glycosylation is considered a hallmark of malignant transformation, and has been observed for almost 20 years [28,31,33,34]. In particular, *O*-glycans play important roles in the attachment and invasion of cancer cells, as well as in their survival in the blood stream [35]. Tumour-associated glycans can be found in the surface of cancer cells, and therefore are easily accessible to antibodies and lectins [47,48]. Moreover, they are released to the peripheral circulation, either in secreted glycoproteins or by shedding from cell surfaces – these features allow them to be explored in various serological assays [33,47]. As such, the presence of tumour-associated glycans in serum is used for post-surgical follow-up, to determine disease recurrence, progression and response to therapeutics [49].

Among the most common structural features associated with cancer are the altered expression of terminal structures, which includes loss of ABO(H) determinants by secretor individuals and changes of Lewis antigenic patterns [33,35]. Moreover, mucins over-expression [50,51], particularly MUC1, and the incomplete *O*-glycosylation, yielding low molecular weight *O*-glycans, namely T and STn, are also associated with the malignant transformation of cells [40,33].

The first evidences of alterations in glycosylation patterns of tumour cells concerns the loss of ABO(H) blood group determinants in gastric cancer [52]. Then, the correlation between the degree of A/B determinants deletion, invasion and metastatic spread was described in lung [53] and head and neck [54] carcinomas. Blood group antigens are known to be involved in several cellular activities, such as cell adhesion, cell recognition and cell-cell signaling [27].

Lewis blood group antigens are related carbohydrates structures used as markers of cell differentiation. In adults, they are present in erythrocytes as well as in different tissues and organs. It has been shown that neoplastic transformation is

often associated with characteristic changes in the expression of these blood group oligosaccharides, and their amounts usually increase during tumour progression and acquiring of malignant phenotype [55].

Changes in Lewis antigenic patterns are related to over-sialylation of terminal structures resulting in an over-expression of SLea and SLex antigens. The referred alterations in glycosylation stem come from an aberrant expression of the genes encoding sialyl and fucosyltransferases [28,30]. Since 1980s several studies begun to demonstrate the role of SLea and SLex expression in cancer specimens, using monoclonal antibodies [56].

The presence in tumour cells of SLea or SLex, which are ligands for selectins, promotes the metastatic process by facilitating the interaction of cancer cells with the endothelium of distant organs, been demonstrated to be correlated with poor prognosis [40,57]. According to Takada *et al.* (1993) SLea antigen is mainly responsible for adhesion of human colon, pancreas and gastric cancer cells to the endothelium, whereas binding of lung, liver and ovarian cancer cells is mediated by SLex [58]. The loss of A and B antigens increases cellular motility, while the presence of O(H) epitopes increases resistance to apoptosis by mechanisms that remain to be defined [57].

Sialylated Lewis (SLe) antigens can also be released into the bloodstream by malignant cells – thus, soluble forms of these antigens are also expressed in high amounts in the blood of many cancer patients [33,34]. Studies based on the average survival of patients with gastric carcinomas after surgery revealed that high levels of SLea and SLex correlated with lower survival [59,60]. Based on these features, SLea and SLex are currently used in non-invasive assessment of tumour progression and metastatic spread [28,33,38].

Normal epithelial cells derived from different organ sites express a subset of the more than 20 mucin core proteins, which are heavily *O*-glycosylated in a specific manner to the requirements of the epithelial cell surfaces in that organ. The process of transformation to malignant state results in the expression of different mucin core proteins with distinct patterns of complex *O*-linked glycosylation, principally in the tandem repeat domain. In cancer includes short, truncated structures not seen in normal epithelia. Of these shortened structures, the most

notable are the pan-carcinoma structures STn and Tn along with *core* 1 glycan extension, the T antigen [61].

Mucins have several functions like protection and lubrification, forming a selective barrier in epithelial surfaces. However, they can be found in more complex biological processes, as epithelial regeneration and differentiation, cellular adhesion and signaling [62-64].

Mucins are the major epithelial luminal surface glycoproteins and are characterized by their high molecular masses (> 200 kDa) and content of carbohydrate side chains (~90%) [65].

The over-expression of mucins during neoplastic transformation, namely MUC1 mucin, has been widely documented [50,51,66].

MUC1 is a transmembrane glycoprotein with a large extracellular mucin-like domain, formed by 30-90 repeats of 20 homologous amino acids rich in *O*-glycosylation sites [67,68]. In carcinomas, MUC1 is expressed over the entire cell surface, in abnormally large amounts, opposing to the expression on the apical domain of normal epithelial cells [33,51]. Since the extracellular domain of MUC1 is long, dense and relatively rigid, due to the abundance of *O*-glycan oligosaccharides, adhesion molecules present in cell surface, such as cadherins and integrins, become shielded. Thus, cell-cell and cell-extracellular matrix interactions decrease, creating an anti-adherent effect [51,69]. Consequently, MUC1 has the capability to induce detachment of a cell from the primary lesion, leading to invasion and metastasis. This process escapes from immune surveillance, because MUC1 inhibits the interaction between cytotoxic lymphocytes and the target cell and promotes apoptosis of lymphocytes, allowing the detached cell to survive in the bloodstream or in distant organs [33,69]. In addition, the concomitant expression of SLex or SLea facilitates the adhesion of tumour cells to the vascular endothelium, promoting metastasis [38,40] Over-expression of MUC1 is generally correlated with higher aggressiveness and metastatic capability of the tumour [51,69].

The sialylation of T (ST) and Tn (STn) antigens is the product of abnormal glycosylation, as explained above. In fact, these antigens expressed in many primary human carcinomas such as ovarian, esophagus, gastric, colon, head and neck, breast, carcinomatous mesothelium, bladder, pancreas and cervix, whereas very low levels or no expression are found in healthy tissues [30,39,66,70-76].

Therefore, ST and STn antigens have been extensively studied in last decades and are widely assumed as pan-carcinoma biomarkers [39,34,77].

T antigen is also considered a pan-carcinoma antigen, once it is substantially over-expressed in several carcinomas, namely breast, bladder, colon, gastric and prostate, being associated with a worse prognosis [30,72,74,78-81]. Nevertheless, T antigen is also expressed in normal tissues although in low levels, because at some time it undergoes further glycosylation [72,78,79]. The alterations in glycosylation patterns during malignancy are often the result of altered activities of sialyltransferases in cancer cells. This is evident in the specific and preferential display of certain glycoconjugates, like ST or STn on cancer cells [30,38]. As previously described, the upregulation of sialyltransferases and consequent increase in the sialic acid content usually blocks further glycosyltransferase activity and chain elongation [35,40]. Consequently, neoplastic cells express heavily sialylated and truncated *O*-GalNAc glycans, namely ST and STn antigens, which have an increased ability to bind to adhesion molecules present in endothelial cells, such as selectins [35,39,40]. Therefore, these highly sialylated *O*-glycans have an enormous invasive potential and metastasis capability [35,39].

The Golgi apparatus disorganization can also affect the glycan content of cells. For instance, in normal cells, the GlcNac-transferases responsible for initiation of core 1 and core 2 *O*-glycans are localized in the *cis*-Golgi, whereas in some cancers they have been found throughout all Golgi compartments [82]. Gill *et al.* (2010) also suggested a redistribution of ppGalNAc-Ts involved in *O*-glycosylation induced by the activation of Src kinase [42]. This activation promotes a relocation of the enzymes from the Golgi apparatus to the Endoplasmatic Reticulum, allowing them to be more time in physical contact with potential substrates. Thus, the occurrence of *O*-glycosylation is dramatically increased, resulting in an enhanced synthesis of truncated *O*-GalNAc glycans [42].

Finally, another possible mechanism underlying the expression of cancer-associated *O*-glycans, suggested by Ju *et al.* (2002) [83], focus on a mutation on the molecular chaperone *Cosmc*. This mutation causes loss of C1β1-3Gal-T activity and may contribute to expression of Tn antigen in tumor cells [83].

Sialyl Tn – a pan-carcinoma antigen

STn detection among healthy tissues is heterogeneous [77,84]. According to Cao *et al.* (1996) the expression of this antigen was found to be positive in normal submandibular glands, sweat gland ducts and in gastric cells, but not in the esophagus, in jejuno, ilieum and gall bladder [84]; moreover, STn can be detected in colonic cells after the removal of *O*-acetyl groups [85], and it was never detected in normal liver (except bile ducts) and pancreas [84,85]. Focusing the urogenital tract, the STn antigen was found in the interstitial cells of the testis, in some uterine and cervix cells, but not in the ovarian neither in urinary bladder [77,84]. Finally, it was also detected in some cells of normal lung tissue, but not in the respiratory epithelium [84]. According to these authors, the expression of STn antigen in normal tissues is rare and/or low, when compared to cancer tissues [84]. Moreover, these studies evidence an expression of STn restricted to secreting cells, which suggest that the spread expression of this antigen in healthy tissues relates to external fluids of the body [77].

As mentioned above, STn has been reported to be neo- or over-expressed in more than 80% of human carcinomas [30,39,70,71,75]. Therefore, STn can be considered a pan-carcinoma antigen and a good tumour marker of carcinogenesis [77]. In line with these observations, several *in vitro* and *in vivo* studies have associated STn antigen with aggressive cell phenotypes [30,39,77]. It has been demonstrated that STn expression promotes major alterations on the cell surface glycosylation profile, inducing or preventing the recognition by lectin like molecules [39]. This process contributes to a malignant phenotype, decreasing cell-cell aggregation and increasing extracellular membrane adhesion, migration, invasion and metastasis [39,77].

In gastric cancer there is still no consensus about STn tissue immunoexpression prognostic value. However, according Nakagoe *et al* (2001) [87], pre-operative serum levels of STn correlate with advanced tumour stages and poor outcome, particularly in stage III/IV gastric cancers [87]. Other investigators, reported a decreased overall survival of the patients with more than 35% esophageal cancer STn-positive cells, when compared to the low expressing group (<35% of STn-positive cells) [88]. Ozaki *et al.* (2012) described that STn-positive gastric cancer cells have demonstrated higher intraperitoneal metastatic ability in comparison

with STn-negative controls, in nude mice, resulting in increased invasion and decreased survival [89]. Moreover, Ogata *et al.* (1992) have previously demonstrated that mucins bearing STn antigen are effective inhibitors of natural killer (NK) cells cytotoxicity [90]. Thus, it was suggested that STn over-expression in cancer specimens induces impairment in NK cells function of the immune system and, subsequently, that mucins expressing the STn antigen allow cancer cells to escape from immune surveillance [90].

In fact, raised levels of serological STn have been associated with decreased overall survival of patients with gastric, esophagus, ovarian and colorectal cancers [28,77,87,91-93]. Given its biomarker value, several authors, according to their studies, indicate the need for STn to become a tumour marker in serological assays (CA 72-4) [91-93]. This antigen is present in the bloodstream due to *O*-glycoprotein secretion or shedding from tumours, which occurs only when the tumour reaches a critical mass. Therefore, the presence of STn in serum is usually detected in advanced tumours, being STn considered a poor prognosis marker [77].

Due to their widespread presence on the cell surface of human tumours, several structurally similar blood group–related carbohydrate antigens attached glycoproteins, in which STn is included, are promising targets for vaccine therapy.
According to Gilewski *et al.* (2007), the development of a STn-based vaccine requires consideration of several variables. The first concerns the natural source of STn (from ovine submaxillary mucin or human cells) or synthetic sources that can influence the reactivity of monoclonal antibodies. The second one refers the low immunogenicity of STn because it is a carbohydrate as well as a ‘‘self-antigen’’ [94]. Several authors refer that one approach to increase its immunogenicity is its conjugation with the keyhole limpet hemocyanin (KLH) immunogenic protein carrier, as well as the addition of immunologic adjuvants such as QS-21 [94,95]. The third refers the possibility that the conformation of STn found on naturally occurring mucins may be different from synthetic STn [94]
A cancer vaccine named *Theratope*, comprehending a synthetic STn disaccharide coupled to a KLH, was developed [96,97]. This cancer vaccine showed some promising results in animal models and phase II trials for breast and ovarian cancers. Briefly, these tests showed that this antigen was safe and produced a

strong immune response against these tumours [98,99]. Particularly, the efficacy of STn-carrying immunogens was investigated in a MUC1 transgenic mouse model. The authors reported that *Theratope* induced anti-STn antibodies that recognized this glycan in a number of glycoproteins. Moreover, a significant delay in tumour growth was observed in these mice, and the protection effect seemed to be dependent on the STn being expressed by the tumour [99]. In spite of all these promising preliminary results, *Theratope* failed phase III clinical trials for metastatic breast cancers, since it did not improved the endpoints of time-to-disease progression and overall survival rate, which was largely because it cannot induce strong T-cell-mediated immune response in patients [100,101]. In a recent review, Julien *et al.* (2012) discussed that the failure of *Theratope* was also related with the design of the study, namely that it disregarded the fact that only 20-30% of the patients with metastatic breast cancers expressed the STn antigen [77]. Nevertheless, the notion prevails that STn based therapeutics may constitute a strategy to control invasion and metastasis, and consequently improving poor outcomes. The fact that it is a cell-surface antigen that may be more easily accessible to antibodies and/or other ligands also offers potential in the context of guided therapeutics.

1.3. Glycosylation and bladder cancer

According to Ohyama (2008) glycosylation status during carcinogenesis and progression, invasion and metastasis in bladder cancer has been well documented [27].

ABO Group antigens

Blood group antigens have long been recognized as key molecules in cell recognition, adhesion, and signaling [102]. The loss of ABO blood group gene transcripts has been related with advanced stage bladder carcinomas [103]. These findings corroborate Thorpe *et al*. (1983) and Summers *et al*. (1983) which had also correlated the loss of ABO antigens with the invasive potential of tumours years before [104,105]. Moreover, Limas *et al*. (1985) have also associated this

event with high grade BC [106]. However, according to Ohno (2014) and Klatte *et al.* (2014), ABO loss might have no significance in superficial bladder tumours [107,108].

Although ABO loss was shown to be associated with advanced and metastatic bladder tumours, little is known about the relation between ABO type and prognosis of patients undergoing radical cystectomy [109,110]. In fact, a better survival outcome was seen in O blood type patients of pancreas and breast cancers [111,112], but no specific type of blood group was statistically associated to bladder tumour patient's outcome [109,110].

Lewis antigens

Several authors have studied the expression of the Lea antigen in bladder tumours. Cordon-Cardo *et al.* (1988) referred that there were no alterations in this antigen expression patterns with malignant transformations in the bladder [113]. However, Limas *et al.* (1985) had already reported significantly lower expression of Lea in healthy urothelium (6%), when compared to invasive tumours of the bladder (35%) [106]. Also Juhl *et al.* (1986) found a high expression of Lea antigen in invasive bladder carcinomas (93%) [114]; both studies strongly suggest that the expression of Lea is associated with a malignant phenotype of bladder tumour [114, 106].

The sialylated form of Lea, the SLea antigen or Ca 19-9, commonly used in pancreatic cancer as a serum marker [115] has been observed in bladder dysplasia, CIS, non-invasive and invasive carcinomas of the bladder [116]. With the purpose of diagnose urothelial carcinoma of the bladder by measuring Ca 19-9 level in the urine, Pal *et al.* (2011) studied 47 patients with confirmed tumour and 50 controls. When compared with normal urothelium, Ca19-9 urine levels were significantly higher in bladder tumour patients. According to their results, Ca19-9 may be a useful non-invasive test to diagnose the urothelial carcinoma of the bladder [117]. Another study compared Ca19-9 levels in serum and in urine of patients with low grade urothelial bladder tumours. Its findings suggested that urinary CA 19-9 is a better screening parameter with optimum sensitivity and specificity than its serum counterpart for diagnosis of low grade and early stages of transitional cell carcinoma of urinary bladder, being also considered as a

prognostic marker [118]. In contrast with these results, Washino *et al*. (2011) and Hegele *et al*. (2010) did not found Ca 19-9 a useful tumor marker when tested in serum or in tumour. However, they did not exclude its presence in bladder carcinomas [119,120].

Neoexpression of the Lewis X antigen (Lex; which is absent in normal urothelium) is noted in over 85% of urothelial bladder cancers regardless of tumour stage and grade [121]. This corroborates the Cordon-Cardo *et al.* (1988) studies that compared the expression pattern of this antigen in healthy urothelium with invasive carcinomas and CIS [113]. Particularly, the study revealed that none of the healthy urothelium specimens expressed Lex; conversely, this antigen was observed in invasive carcinomas of the bladder (100%) and CIS (79%). Furthermore, the authors suggested that Lex expression could be a reliable indicator of malignant transformation of bladder urothelium [113].

Sialy Lex (SLex), the sialylated form of Lex, has also been studied in bladder urothelium. According to Numahata *et al.* (2002) the SLex antigen is present in invasive tumours but absent in noninvasive ones [122]. Moreover, patients who had mAb SNH3 (an anti-SLex monoclonal antibody) positive tumours had significantly lower survival rates, compared with patients who had SNH3 negative tumours. Futhermore, the SNH3 staining of tumours was not correlated with tumour grade (2 or 3) or pT (pT2-T4) classification but was strongly correlated with the presence of lymph node metastasis at the time of first clinical examination [122]. These findings were corroborated by Kajiwara et al. (2005) that observed altered SLex patterns in all of the invasive carcinomas specimens studied, namely renal pelvis, ureter and urinary bladder. Both studies referred altered SLex expression as a predictor of invasive potential and metastatic outcome [122,123].

Thomsen-Friedenreich related antigens

Increased levels of truncated *O*-GalNAc glycans have also been observed in BC. Of note, only a few reports were published, almost all more than 20 years ago. [74,124]. These studies have demonstrated that T and Tn antigens are over-expressed in bladder carcinoma cells, compared to normal cells which practically do not express these glycans [74,84]. In particular, Limas C. *et al*. (1986) evaluated by immunohistochemistry the expression of the T antigen in biopsies

from 56 BC patients at various stages of the disease, including normal and pre-neoplastic bladder disease. These authors demonstrated a growing expression of T antigen through healthy (0%), pre-neoplastic (14%) and neoplastic (11-65%) urothelium [125]. According to their results the spontaneous expression of T antigen is associated with the aggressiveness of the tumour; being this expression correlated with a greater metastatic potential [125]. Tn, STn, T and cryptic-T (ST comprising S6T, S3T and DST) antigens were evaluated by Langkilde *et al.* (1992) in patients with initially non-invasive carcinomas whom experienced different courses of the disease [126]. No association was found between Tn and STn antigens and tumour progression to invasion. Nevertheless, 70% of the patients that expressed T antigen and 39% of those whom expressed cryptic-T antigen experienced invasive recurrence, while the other patients did not [126]. The same authors, few years later studying primary non-invasive and superficially invasive human urinary bladder tumours reinforced the idea that there is a correlation between T-antigen expression and recurrence or progression of initially non or superficially invasive tumours [127].

Altogether, these findings suggest that BC patients whom spontaneously express T antigen present invasive recurrences more frequently than those who do not express this antigen. Furthermore, T antigen expression seems to correlate with the metastatic potential and aggressiveness of the tumour [125-127].

Yamada *et al.* (1988) have demonstrated that cryptic T (ST) antigen is normally expressed by noninvasive or superficially invasive (Ta or T1) urothelial papillary tumours [128]. These authors reported that ST expression was partially or completely lost in tumours that showed malignant progression after frequent recurrences. More recently, Videira *et al*. (2009) demonstrated that ST3Gal.I plays a major role in the sialylation of the T antigen in BC [74]. According to this study, the overexpression of ST3Gal.I seems to be part of the initial oncogenic transformation of bladder cells and should be considered when predicting cancer progression and recurrence [74].

The studies presented so far suggest that both pre-neoplastic lesions and bladder tumours express altered glycosylation patterns. Some studies also point out that alterations in cell glycosylation patterns are generally associated with tumour grade, invasion and metastasis. Therefore aberrant glycosylation correlates with poor prognosis [122,125-128]. Following this thought, targeting these antigens

may allow the determination of the malignant potential of the tumour and the control of the disease. Nevertheless, these studies have been performed in small and heterogeneous patient cohorts using different antibodies. As such, a careful interpretation of the results should be conducted, since several antibodies have affinity for similar structurally-related glycosylated structures [122,129]. Different methodologies have been used, which may also contribute to biased interpretations.

Sialyl-Tn

Despite the cancer-associated nature of the STn antigen, few studies have been presented in the context of BC. Langkilde *et al.* (1992) conducted the first study involving this antigen on a series of high-grade urothelial cell carcinomas, using normal mucosal specimens of patients with non-malignant bladder urologic diseases as controls [126]. The authors reported that STn was not expressed by the control group and showed a very restricted pattern of expression in bladder tumours. Moreover, no association with recurrence and progression was observed [126]. However, four years later, *in vitro* studies have demonstrated that STn expression enhanced the invasion capability of bladder cancer cells [130].
Still, little information is available about the biological and clinical significance of STn expression in BC. In spite of other tumour types, there are no consistent studies in bladder that demonstrate its association with invasion and metastasis. As such it is of prime importance to investigate the relevance of this antigen in BC models and to encourage the development of therapeutics targeting this antigen.

2. AIMS and STUDY OUTLINE

Based on previous knowledge, the main objective of this thesis is to understand the biological and clinical significance of sialyl-Tn expression in bladder cancer, aiming to improve patient stratification and guide the design of novel therapeutics. In order to accomplish it, STn was studied in three dimensions: relating it with the clinical outcome, with the tumour treatment and with tumour biology.

The specific aims of this thesis are:

- Evaluate the pattern of STn expression in bladder tumours and its association with clinicopathological variables and disease prognosis, envisaging to improve patient stratification;
- Determine the biological significance of STn expression in bladder cancer, namely in cell proliferation, invasion and migration;
- Evaluate the role of STn in the context of BCG immunotherapy outcome, generally used as adjuvant therapy in patients with non-muscle invasive tumours showing a high risk of progression to muscle-invasion;
- Determine associations between STn expression and the activation of the PI3K/Akt/mTOR cascade, associated with poor prognosis in bladder cancer.

To accomplish the first objective, a preliminary study was performed in small series of UBC (69 patients), of several grades and stages. STn was accessed by immunohistochemistry. Furthermore, a glycoengineered cell model expressing STn was developed and used to access the role of the antigen in cell motility and invasion. The results are presented on Chapter 3.1, in Paper I entitled “Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours”.

After the previous results (Paper I), and because STn is known to influence both cell-cell interactions and immune response, it seemed to be important to evaluate the influence and the predictive value of this antigen in the context of BCG immunotherapy. Regarding the evaluation of the tumour-associated carbohydrate antigen STn role in BCG immunotherapy, the group work is presented in Chapter

3.2, in Paper II, entitled “Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy”.

Both previous findings resulted in the final objective that was achieved when STn status was related with other biological markers like the ones involved in the mammalian target of rapamycin (mTOR) cascade. The mTOR signaling cascade is a key pathway in the progression of different cancers. In bladder cancer, several groups showed that mTOR activation correlated with reduced disease specific survival and increased pathologic staging [131]. In Chapter 3.3, the influence of STn in the mTOR cascade, as well as its possible importance in bladder cancer discrimination of potential response to a subset of therapeutics was described in Paper III, entitled “Abnormal protein glycosylation and activated PI3K/Akt/mTOR pathway: role in bladder cancer prognosis and targeted therapeutics”.

3. MATERIAL & METHODS, RESULTS and DISCUSSION

3.1- Sialyl Tn expression and advanced bladder tumours

Paper I

Overexpression of tumour-associated carbohydrate antigen Sialyl-Tn in advanced bladder tumours

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Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours

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ABSTRACT

Little is known on the expression of the tumour-associated carbohydrate antigen sialyl-Tn (STn), in bladder cancer. We report here that 75% of the high-grade bladder tumours, presenting elevated proliferation rates and high risk of recurrence/progression expressed STn. However, it was mainly found in non-proliferative areas of the tumour, namely in cells invading the basal and muscle layers. STn was also found in tumour-adjacent mucosa, which suggests its dependence on a field effect of the tumour. Furthermore, it was not expressed by the normal urothelium, demonstrating the cancer-specific nature of this antigen. STn expression correlated with that of sialyltransferase ST6GalNAc.I, its major biosynthetic enzyme. The stable expression of ST6GalNAc.I in the bladder cancer cell line MCR induced STn expression and a concomitant increase of cell motility and invasive capability. Altogether, these results indicate for the first time a link between STn

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Tumour-associated glycans
Proliferative bladder cancer

expression and malignancy in bladder cancer. Hence, therapies targeting STn may constitute new treatment approaches for these tumours.

1. Introduction

Bladder cancer, the fifth most common cancer in Western society, is a growing concern, owing to increased incidence during the past years (Ploeg et al., 2009; van Rhijn et al., 2009). Most of the newly diagnosed bladder cancer cases are superficial, or low-grade non-muscle invasive papillary tumours, being conservatively treated by complete transurethral resection of the tumour (Babjuk et al., 2012). However, approximately half of the patients show a high-percentage of recurrences and an elevated risk of progression to muscle invasive disease, which correlates with poor prognosis (Hussain et al., 2009). The risk of recurrence and/or progression is mostly determined by clinicopathological features (Babjuk et al., 2012). According to the European Organization for Research and Treatment of Cancer (EORTC), this group includes high grade (HG) papillary tumours and carcinoma *in situ* (CIS) and those with multifocal or recurrent lesions (Babjuk et al., 2012). The evaluation of the nuclear protein Ki-67 (Ki-67 proliferation index), an established marker of cell proliferation, is often used to enhance the prognostic accuracy of risk classification given by clinicopathological features (Margulis et al., 2009; Santos et al., 2003), since it is considered a surrogate biomarker of bladder cancer aggressiveness, disease recurrence and progression (Margulis et al., 2009; Santos et al., 2003).

Tumour resection followed by a schedule of intravesical instillations with live attenuated strains of *Mycobacterium bovis* (Bacillus Calmette–Guérin, BCG) is the standard adjuvant therapeutic option for high-risk of recurrence/progression bladder tumours (Askeland et al., 2012; Babjuk et al., 2012). Although BCG has improved the management of high-risk patients, 30–40% of cases either show intolerance or relapse after treatment (Yates and Roupret, 2011). Consequently, these patients require life-long follow-up and repeated courses of treatment making bladder cancer the costliest to treat among solid tumours (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Upon therapeutic failure and/or muscle invasion, cystectomy is advocated for oncological control (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Furthermore, at the moment there is a lack of specific biomarkers to target aggressive cell phenotypes and direct molecular-based therapy, which may be used to avoid preventive cystectomy (Dovedi and Davies, 2009).

Vaccines using tumour-associated glycans, in association with immunological boosters, are emerging as potential therapeutic strategies against cancer (Hakomori, 2001; Lakshminarayanan et al., 2012; Ryan et al., 2010; Sorensen et al., 2006). In the forefront of these antigens is sialyl-Tn (STn; Neu5Acα2-6GalNAcα-*O*-Ser/Thr) (Gilewski et al., 2007; Julien et al., 2009; Miles et al., 2011). STn has been mostly observed in tumour-associated mucins due to their high number of potential *O*-glycosylation sites (Clement et al., 2004; Conze et al., 2010; Julien et al., 2006; Marcos et al., 2011; Pinto et al., 2012). However, integrins (Clement et al., 2004) and CD44 (Julien et al., 2006), among other proteins, may also carry this posttranslational modification. Overexpression of STn antigen has been detected in breast (Leivonen et al., 2001), oesophagus (Ikeda et al., 1993), colon (Itzkowitz et al., 1989), pancreas (Kim et al., 2002), stomach (David et al., 1996; Marcos et al., 2011), endometrium (Inoue et al., 1991), and ovary (Numa et al., 1995) carcinomas, whereas low or no expression was observed in the respective normal tissues. STn overexpression was also reported in several cancer precursor lesions, such as esophageal dysplastic squamous epithelia (Itoh et al., 1996), gastric intestinal metaplasia (Baldus et al., 1998; Ferreira et al., 2006) and colonic moderate dysplasia (Cao et al., 1997).

STn is known to influence cell recognition by the immune system (Angata et al., 2007), affect processes as cell cycle, apoptosis, and actin cytoskeleton dynamics, decrease cell–cell aggregation and increase extra-cellular adhesion, migration, invasion (David et al., 1996; Julien et al., 2006, 2005; Pinho et al., 2007) and metastization (Ozaki et al., 2012). In line with these observations, STn positive (STn^+) cells have been frequently observed at the invasion front of tumours and in peritoneal and pleural effusions in ovarian cancer patients; yet they are less common in metastatic lesions than in primary tumours (Davidson et al., 2000). In gastric carcinomas, STn was correlated with the depth of invasion and metastization (Ikeda et al., 1993), and thus poor prognosis (Terashima et al., 1998). Conversely, STn was not correlated with the depth of invasion in studies concerning colorectal (Itzkowitz et al., 1989; Ogata et al., 1998) and breast cancers (Schmitt et al., 1995). However, some contradicting results have been presented regarding its association with metastasis and decreased survival in these cancers (Julien et al., 2012). Hence, a recent review suggests that the biological role of STn in tumour development may be dependent on each cancer type or sub-type (Julien et al., 2012).

Despite these observations, there is little information regarding STn in the context of bladder cancer. Given its clinical relevance and the fact that there are available therapies based on this antigen, we addressed the presence of STn in bladder tumours and the mechanisms underlying its expression.

2. Materials and methods

2.1. Patient and sampling

Formalin-fixed, paraffin embedded (FFPE) tissues were prospectively collected from 69 patients, mean age of 69 years (age range 45–89), who underwent transurethral resection (TUR) of the bladder tumour in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011

and May 2012. Based on urothelial carcinoma grading and staging criteria of the World Health Organization (WHO), three different groups were considered (Table 1), low-grade (LG, $n = 24$) and high-grade HG non muscle-invasive (NMIBC, $n = 26$) and muscle-invasive (MIBC, $n = 19$) bladder cancers. Of HG NMIBC, 21 were papillary tumours and 5 were carcinoma *in situ* (CIS). None of these patients had received prior adjuvant therapy. Six normal urothelium tissues of necropsied male individuals without bladder cancer history, within the same mean of age range, were also included.

Additionally, FFPE tissues from 16 radical cystectomy cases including the main lesion in each specimen, responsible for therapeutic decision, the adjacent mucosa, which may or may not include a concomitant tumour, and the ureter representing a distant mucosa, were also studied. Mucosa without visible histopathological alterations was defined as "histologically normal" mucosa.

All procedures were performed under the approval of the Ethics Committee of IPO-Porto, after patient's informed consent. All clinicopathological information was obtained from patients' clinical records.

2.2. Tissue expression of STn and Ki-67

FFPE tissue sections were screened for STn and Ki-67 by immunohistochemistry using the avidin/biotin peroxidase method. Briefly, 3 μm sections were deparaffinised with xylene, rehydrated with graded ethanol series, microwaved for 15 min in boiling citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 min. The expression of STn was then evaluated using anti-STn mouse monoclonal antibody, clone TKH2 (Kjeldsen et al., 1988), that identifies both single and clustered STn residues (Ogata et al., 1998), whereas Ki-67 was evaluated using monoclonal mouse anti-human Ki-67 antibody, clone MIB-1 (Dako). After blockage with BSA (5% in PBS), the antigens were identified with Vectastain Elite ABC peroxidase kit (Vector Lab) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the slides were counterstained with haematoxylin for 1 min. Positive and negative control sections of intestinal metaplasia were tested in parallel. The negative control sections were performed by adding BSA (5% in PBS) devoid of primary antibody. STn^+ tissues were also treated with a neuraminidase from *Clostridium perfringens* (Sigma–Aldrich) as previously described by Marcos et al. (2011) in order to remove the sialic acid. The desialyated samples were thereafter screened for STn. The *O*-acetylation of Neu5Ac residues in STn was evaluated after treatment with 100 mM NaOH at room temperature for 30 min as described by Ogata et al. (Ogata et al., 1998) prior to immunohistochemistry with antibody TKH2.

A semi-quantitative approach was established to score the immunohistochemical labelling based on the intensity of staining and the percentage of cells that stained positively. The STn and Ki-67 expression were assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached. Tumours were classified as proliferative whenever Ki-67 expression was higher than 18%, as described by Santos et al. (Santos et al., 2003).

Table 1 – STn expression in the healthy urothelium and in non-muscle invasive (NMIBC) and muscle invasive (MIBC) bladder cancers of different clinicopathological natures.

	Total	STn expression
Normal urothelium	6	
–		6 (100%)
+		–
++		–
+++		–
Total STn^+		0 (0%)
NMIBC	50	
Low-grade papillary tumours	24	
–		19 (79%)
+		5 (21%)
++		–
+++		–
Total STn^+		5 (21%)
High-grade (CIS + papillary tumours)	26	
Carcinoma in situ (CIS)	5	
–		4 (80%)
+		1 (20%)
++		–
+++		–
Total STn^+		1 (20%)
High-grade papillary tumours	21	
–		5 (24%)
+		9 (43%)
++		4 (19%)
+++		3 (14%)
Total STn^+		16 (76%)
MIBC	19	
–		5 (26%)
+		11 (58%)
++		2 (11%)
+++		1 (5%)
Total STn^+		14 (74%)

–: No reactivity; +: ≤15%; ++: 15–30%; +++: 30–45% of the tumour.

2.3. Cell lines culture

The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and $MCRSTn^+$), were grown as described by Videira et al. (2009b).

2.4. Generation of STn^+ bladder cancer cells

MCR cells were transduced with a retroviral vector generated with the ViraPower™ Lentiviral Expression System (Invitrogen), according to manufacturer's instructions. The whole coding region of human ST6GalNAc.I was PCR amplified and cloned in the pLenti6/V5 Directional TOPO cloning vector which drives the expression of inserted genes through the CMV promoter. A negative control retroviral vector was prepared with an empty plasmid. After transduction with negative control- or ST6GalNAc.I-expressing vectors, MCR cells were selected with 4 μg ml^{-1} blasticidin. An additional immunomagnetic enrichment of the STn^+ cells was performed by using mouse anti-STn (HB-STn1 clone from Dako), followed by the secondary antibody anti-mouse IgG associated to paramagnetic microbeads (Miltenyi Biotec). The stable transduction of the enzyme was confirmed by evaluation of ST6GalNAc.I expression and activity. STn expression was

determined by analysis of the mean fluorescence intensity (MFI) ± SE through flow cytometry analysis using monoclonal antibody TKH2.

2.5. Evaluation of STn expression in cell lines

For phenotypic characterization, cells were stained with 1:50 diluted anti-STn TKH2 monoclonal antibody for 16 h at 4 °C, and 1:100 diluted goat fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Dako) for 15 min at 4 °C in the dark and then acquired in a FacsCalibur Flow cytometer (Becton Dickinson). Data were analysed using the WinMDI v2.9 software (The Scripps Research Institute, San Diego, CA, USA).

2.6. Analysis of ST6GalNAc.I expression

RNA extraction from FFPE sections was performed after deparaffinization of the tissue using Absolutely RNA FFPE kit (Agilent technologies) while for cell lines it was used the GenElute Mammalian Total RNA Purification kit and DNAase treatment (Sigma), according to the manufacturer's instructions. The purity of RNA extracts was determined based on the A_{260}/A_{280} ratio. Only ratios between 1.9 and 2.1 were considered further.

Approximately 250–500 ng of total RNA (1 μg for cell lines) was converted by reverse transcription into cDNA, using the random-primers-based High Capacity cDNA Archive Kit (Applied Biosystems). The expression levels of ST6GalNAc.I were determined by TaqMan assay (Applied Biosystems), the reference sequences detected by each primer/probe set and the Assay ID provided by the manufacturer were the following: ST6GalNAc1 (NM018414.2/Hs00300842_m1). Real time PCR was performed in a 7500 Fast Real-Time PCR System using the TaqMan Universal PCR Master Mix Fast from Applied Biosystems, as described previously by Videira et al. (2007, 2009a). During the cDNA exponential amplification the product formation was proportional to the fluorescence emission resulting from the TaqMan probe degradation (van der Velden et al., 2003). The ST6GalNAc.I mRNA levels were normalized for the expression of β-actin, which was taken as a suitable endogenous control for bladder cancer cells (Videira et al., 2007). The relative mRNA levels were calculated by adapting the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

2.7. Evaluation of ST6GalNAc.I activity

MCR cell pellets were homogenized in H_2O and the protein concentration was determined using the RC-DC protein quantification kit (BioRad) according to the manufacturer's instructions. Sialyltransferase activity was assayed in whole cell homogenates as previously described by Dall'Olio et al. (1997) with some modifications. Briefly, the reaction mixture contained 80 mM sodium cacodylate buffer pH 6.5, 0.5% Triton X-100, 6 μg μl^{-1} of asialo bovine submaxillary mucin (ABSM, prepared by acid desialylation of BSM) as acceptor substrate, 30 μM (1280 Bq) of CMP-[^{14}C]Sia (Amersham) and 2 μg μl^{-1} of homogenate proteins. Endogenous controls were prepared in the absence of acceptor substrate. The enzyme reactions were incubated at 37 °C for 2 h and the acid insoluble radioactivity was measured as previously described by Dall'Olio et al. (1997). The incorporation on endogenous substrates was subtracted.

2.8. Cell proliferation measurement

To study their proliferative capacity, cells were labelled with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). The MCR cells were resuspended into medium at final concentration of 1×10^6 cells ml^{-1} and incubated with 10 μM CFSE, following the manufacturer's instructions. Subsequently, the CFSE-labelled cells were seeded into 24-well microplates, incubated in a 5% CO_2 incubator at 37 °C and harvested at 24, 48, 72 and 96 h post-culture. Flow cytometry using a FACSCalibur (Becton–Dickinson) was performed and the data collected were analysed with ModFit LT 3.2 software (Verity Software House, Topsham, ME), allowing to assess the cell proliferation index (PI). The PI represents the average number of cells that were originated from a single cell of the parental generation. The parental generation was set based on the analysis of data obtained from the cells corresponding to the 24 h of culture.

2.9. Analysis of cell motility using a wound-healing assay

Cell motility was tested in a wound-healing migration assay. MCR cells were seed into 12-well microplates and grown to confluency. A scratch was made in the monolayer with a sterile 200 μl pipette tip. After wounding, the suspended cells and debris were washed away and fresh medium was added. At 0 and 24 h after wounding, scratched regions were photographed with an inverted microscope equipped with a digital camera.

2.10. Invasion assay

Invasion assays were performed using BD Biocoat Matrigel™ invasion chambers, comprised by an 8-μm diameter pore size filter coated with a thin layer of matrigel, and placed in a two-compartment system in a 24-well plate. Prior to each experiment, filters were re-hydrated in serum-free DMEM medium for 2 h at 37 °C. After detachment of subconfluent cells with trypsin/EDTA, cells were suspended in culture medium supplemented with 5% inactivated FBS, counted and seeded on the upper side of the matrigel-coated filter at a density of 5×10^4 cells/well. After 24 h at 37 °C, filters were fixed in 4% paraformaldehyde and non-invading cells, present on the upper side, were completely removed, to facilitate analysis. Cells that had invaded the underside of the filters were mounted in Vectashield+4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA), and visualized through a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Germany). Invasive cells were scored in at least 12 microscopic fields (20× objective) when DAPI-counterstained nuclei passed through the filter pores. Results are presented as means ± SD for each sample. Invasion levels are expressed as a ratio of the results obtained with the mock-transfected control cell line.

2.11. Statistical analysis

Statistical analysis was performed using the Student's *T*-test for unpaired samples. Differences were considered to be significant when $p < 0.05$. A chi-square test was used to analyse correlations between clinicopathological features and STn and Ki-67 expressions.

3. Results

3.1. Expression of STn in bladder tumours

STn expression in bladder tumours was evaluated by immunohistochemistry using mouse monoclonal antibody clone TKH2. As shown in Table 1, STn is not expressed in the healthy urothelium; conversely 46% of the bladder tumours presented cells with STn membrane and cytoplasmic staining (32/69) (Figure 1), demonstrating the tumour-specific nature of this antigen. The removal of sialic acids from the tissue sections with a α-neuraminidase impaired the recognition by TKH2 and confirmed STn expression.

STn expression was lower in low-grade (LG) NMIBC (21% STn^+ tumours; Figure 1A–B) compared to high-grade lesions (HG; 67%), which include papillary tumours (76% STn^+ tumours; Figure 1C–E), CIS (20% of STn^+ tumours; Figure 1F), and MIBC (74% STn^+ tumours; Figure 1G–H). Noteworthy, STn was absent from the majority of CIS (4/5; 80%) and showed an expression comparable to LG tumours. Altogether, these results highlight an association between the STn antigen and high grade NMIBC ($p < 0.002$; Figure 2) as well as with muscle invasive tumours ($p < 0.03$; Figure 2).

The *O*-acetylation of sialic acid residues prevents TKH2 from recognizing STn antigens in certain tissues (Ogata et al., 1998). To exclude this possibility in bladder cancer, the slides were chemically de-*O*-acetylated prior to immunohistochemistry. This procedure did not alter STn expression patterns demonstrating that STn antigens were not encrypted by *O*-acetylation.

3.1.1. Pattern and extension of STn expression in bladder tumours

The STn antigen presented a focal expression that for the majority of the STn positive cases (26/36) did not exceeded 15% of the tumour section (Table 1). Furthermore, in 25% of the STn positive cases (9/36) the antigen was detected in less than 5% of the tissue (data omitted from Table 1). Higher expression patterns were restricted to HG papillary NMIBC, where 27% of the cases (7/26) presented STn levels between 15% and 45% of the tumour section (Table 1) and locally diffuse staining (Figure 1C, D, G). STn was mainly observed in basal layer cells (75% of STn^+ cases; Figure 1A, C–E), but it could be also detected throughout the papillae (Figure 1C–E) and cells of the luminal surface (Figure 1F) in cases presenting locally diffuse staining. STn was further observed in cells invading the basal (50% of STn^+ of HG NMIBC; Figure 1C–E, G) and muscle layers (57% of STn^+ MIBC; Figure 1G, H), suggesting a role in invasion.

3.1.2. STn antigen expression in advanced tumours and in the surrounding areas

The STn antigen was also evaluated in a series of radical cystectomy specimens which included the tumour used for therapeutic decision (termed "main tumour" in Figure 3) and the tumour-adjacent mucosa. The ureters were included as distant mucosa (Figure 3). In agreement with the observations from Table 1, STn was detected in 69% (11/16) of all main tumours as well as in their adjacent mucosa (Figure 3), independently of their histological classification. Noteworthy, STn was absent from 90% of the distant mucosas of STn positive cases; the only exceptions being a ureter with pre-neoplastic and another with a neoplastic lesions (Figure 3). These results point out that the STn^+ tumour-adjacent mucosa may display molecular changes similar to those of the main lesions. Thus, this antigen may be useful as a marker of field carcinogenesis in the bladder.

3.2. Expression of ST6GalNAc.I in bladder tumours

The presence of STn has been strongly associated with the overexpression of ST6GalNAc.I in several human malignancies. To assess this event in bladder tumours, mRNA levels of *ST6GalNAc.I* gene were analysed and normalized in relation to β-actin, which proved to be a stable expressed gene in previous studies concerning bladder tumours (Videira et al., 2007). As shown by Figure 4, low gene expression levels were detected in tumours that did not express STn. In addition, the levels of *ST6GalNAc.I* increased with the expression of STn, and were significantly higher in the tumours with STn expression superior to 15%. Figure 4 also shows that this behaviour was similar in LG and HG tumours. However, as a result of higher STn expression, the average ST6GalNAc.I mRNA levels were more elevated in HG (53%) tumours than LG (9%). These observations suggest that overexpression of *ST6GalNAc.I* gene is one of the main events leading to STn expression in bladder tumours.

3.3. STn expression and tumour proliferation

As shown above, the expression pattern of STn correlates with HG tumours, known to present elevated proliferation rates (Margulis et al., 2009; Santos et al., 2003). To assess a possible association between STn and proliferation, 24 cases from the initial series of 69 bladder tumours, comprehending 12 LG and 12 HG tumours (7 NMIBC, none of them CIS, and 5 MIBC), were screened for STn and Ki-67 expression. Tumours presenting Ki-67 expression superior to 18% were classified as proliferative. As highlighted by the graphical matrix in Figure 5A, 8% (1/12) LG and 75% (9/12) HG cases showed elevated Ki-67, confirming the higher proliferation of HG tumours ($p < 0.0012$). Similarly, Figure 5A also shows an association between proliferative phenotypes and STn expression ($p < 0.001$). However, in all STn positive cases, the examination of sequential sections revealed that STn antigen expression was mainly seen in areas that did not express Ki-67 (Figure 5A), although some overlap was present in 25% of the cases (3/12; Figure 5B). This indicates that the STn antigen is mostly expressed in non-proliferative areas of the tumour. Nevertheless, the majority of the non-proliferative tumours also did not express STn (12/14), demonstrating an interdependence between both phenomena.

3.4. In vitro assessment of the biological significance of STn expression

3.4.1. Development of a high-grade bladder cancer cell line overexpressing STn

To further corroborate the role of ST6GalNAc.I in the expression of STn antigen by bladder cancer cells, we induced the overexpression of ST6GalNAc.I in a bladder cancer cell line. The MCR bladder cell line, that showed negligible expression

of ST6GalNAc.I and no STn (data not shown), was transduced with a lentivirus expressing the coding region of the human *ST6GalNAc.I* gene. The obtained cell line variant, herein named MCRSTn$^+$, showed markedly increased expression of *ST6GalNAc.I* mRNA levels (Figure 6A). It also showed significantly higher sialyltransferase activity towards the ABSM, a substrate for the ST6GalNac.I enzyme, when compared with the negative control cell line (MCRnc) transduced with void lentivirus (Figure 6A). The overexpression of STn antigen by MCRSTn$^+$ cell line variant was confirmed by flow cytometric analysis (Figure 6B).

3.4.2. *STn influence on cell proliferation, migration and invasion*

STn expression was correlated with tumours with higher proliferative indexes (Figure 5). To assess the influence of STn in

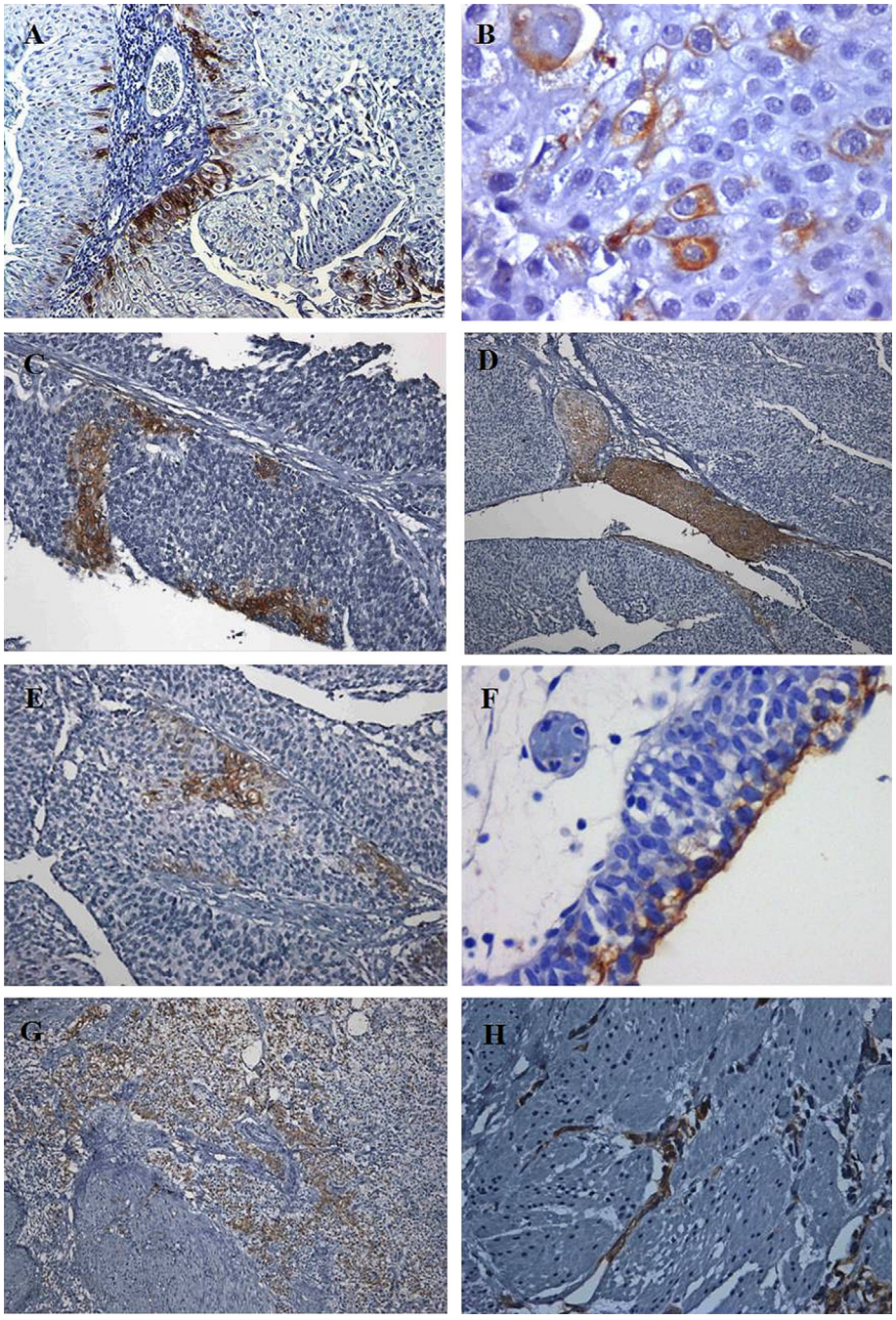

Figure 1 – Expression of STn in FFPE bladder tumours. A) Low-grade papillary tumour showing a predominance of STn$^+$ cells in the basal layer; B) Magnification which shows tumour cells with membrane and cytoplasmic STn$^+$ staining; C) High-grade papillary tumour evidencing the focal nature of STn expression. Positive cells were found both in the basal layer and throughout the papillae; D) High-grade papillary tumour showing locally extensive STn positivity; E) High-grade papillary tumour evidencing STn$^+$ in the basal layer; F) CIS showing STn$^+$ in the cells facing the lumen of the bladder; G) MIBC showing locally extensive STn expression including at the muscle invasive front; H) MIBC highlighting STn$^+$ cells invading the muscle layer.

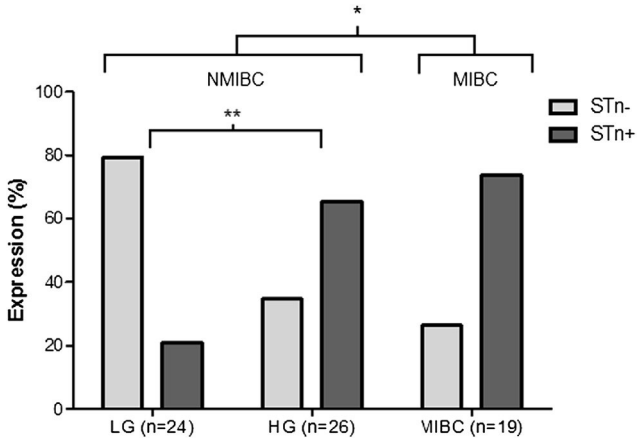

Figure 2 – Association between STn expression and HG NMIBC and MIBC. The percentage of STn$^+$ tumours was higher in HG when compared to LG and also in MIBC when compared to NMIBC (LG + HG). "*" $p = 0.03$; "" $p = 0.002$ (Chi-square test).**

proliferation, MCR cells (MCRnc and MCRSTn$^+$) were cultured for 48, 72 and 96 h and then evaluated in relation to their proliferation index. The comparison between the two cell line variants showed that the proliferation index of MCRSTn$^+$ cells was generally higher than the index of MCRnc cells, although only statistically different at 72 h of culture ($p < 0.05$; Figure 7). However, this effect was no longer significant at 96 h of culture (Figure 7).

STn positive cells were observed invading the basal and muscle layers (Figures 1 and 2) and in the adjacent mucosa of advanced stage bladder tumours (Figure 5), suggesting a correlation of STn with invasion and migration. Thus, the influence of STn expression in MCR cell invasion was assessed using the Matrigel invasion assay. Our results evidence that

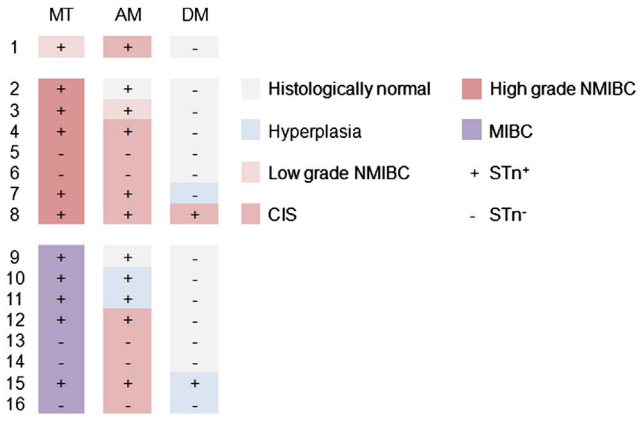

Figure 3 – Expression pattern of STn in radical cystectomy specimens. Radical cystectomy specimens have been organized based on histological grade. They include the tumour responsible by the therapeutic decision termed "main tumour" (MT), an adjacent (AM) and distant mucosa (DM). The graphical matrix highlights that, whenever STn is expressed by the main tumour (13/16; 63%), it is always present in the adjacent mucosa (13/13, 100%). One preneoplastic and one neoplastic distant mucosa also expressed the antigen.

MCR cells transduced with ST6GalNAc.I (MCRSTn$^+$) are approximately four folds more invasive than bladder cells transduced with the negative control (MCRnc; Figure 8A). The effect of STn expression on cell migration was estimated by a wound-healing assay. Therefore, uniform scratches were made in confluent monolayers of MCRnc and MCRSTn$^+$ cell lines and the capability of the cells to migrate and fill the scratches was monitored. As observed in Figure 8A, by 24 h after wounding, the MCRSTn$^+$ cells had almost completely covered the empty space. Conversely, the negative control, MCRnc cells, displayed a large "gap", thus demonstrating their lower capability to closure the wound. Our results evidence that MCR cells expressing STn present increased invasion and wound repair capacities.

4. Discussion

The STn antigen is highly expressed by several human carcinomas and preneoplastic lesions (Julien et al., 2012) and is explored as a tumour marker in serological assays (CA72-4) (Reis et al., 2010).

Despite the clinical relevance of STn in human malignancies, scarce information is available about its role in bladder tumours. Over twenty years ago, Langkilde et al. (1992) addressed this antigen on series of transitional cell carcinomas (currently classified as high-grade urothelial cell carcinomas according to current WHO guidelines (Babjuk et al., 2012)). Normal mucosal biopsy specimens from patients with non-malignant bladder urologic diseases were included as controls. According to the authors, STn was not expressed by the control group, showed a very restricted pattern of expression in bladder tumours and no association with recurrence and progression. Subsequent *in vitro* studies found that

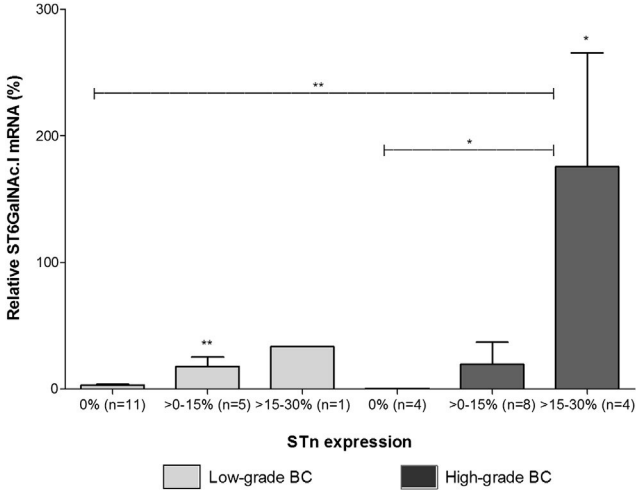

Figure 4 – Association between ST6GalNAc.I and STn expression in LG and HG bladder tumours. The graph shows that ST6GalNAc.I expression is increased in STn$^+$ tumours and increases further for more elevated STn expressions (>15–30% of the tumour section). This suggests that the overexpression of ST6GalNAc.I is one of the main mechanisms underlying the presence of STn in bladder cancers. Furthermore, it shows this event occurs in both LG and HG tumours. "*" $p < 0.05$; "" $p < 0.01$ (Student's *T*-test).**

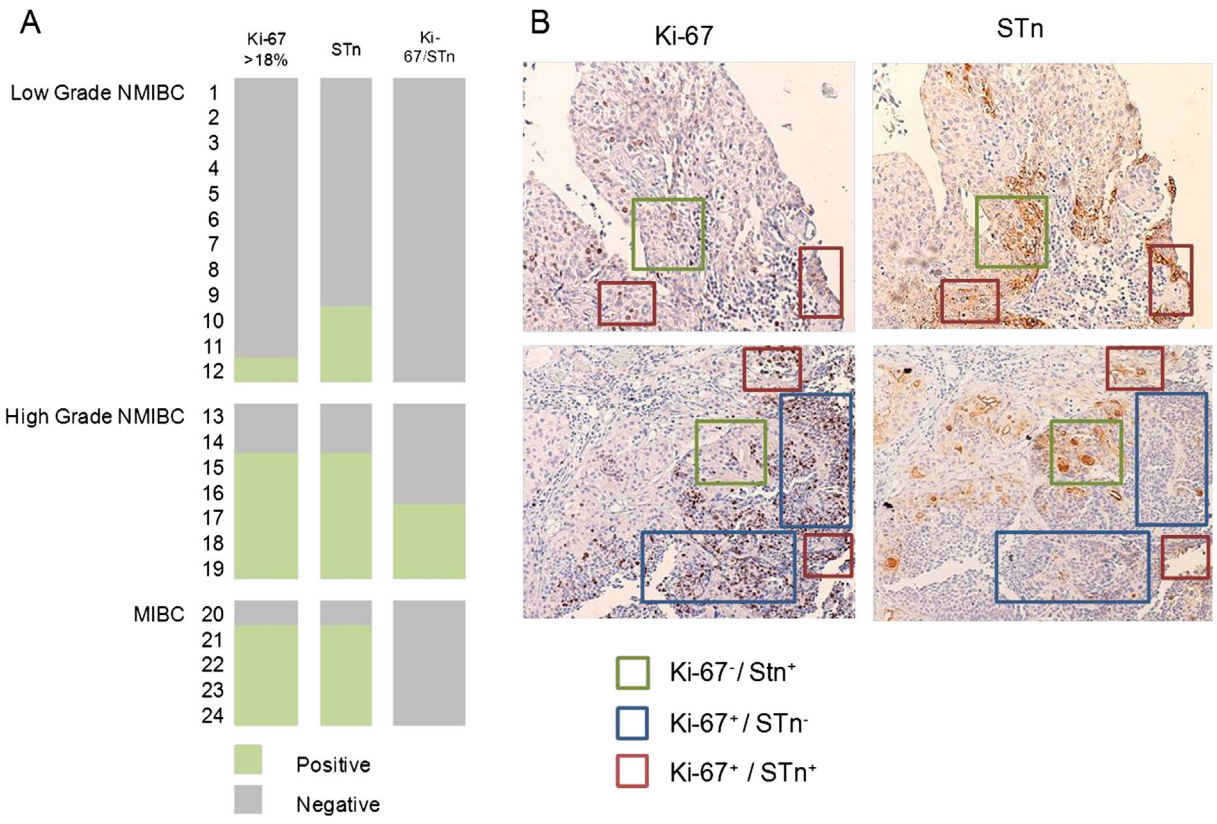

Figure 5 – Expression of STn and Ki-67 in bladder tumours. A) graphical matrix highlighting the association between proliferative tumours (Ki-67 > 18%) and STn expression in bladder tumours. HG NMIBC and MIBC were considered to be proliferative tumours and a significant association was found between STn expression and tumours presenting proliferation phenotypes ($p < 0.001$; Chi-square test). The notation "Ki-67/STn" in the column more to the right refers to tumours presenting areas that appear to exhibit cells expressing both Ki-67 and STn. B) Immunohistochemistry for Ki-67 and STn highlighting Ki-67$^-$/STn$^+$; Ki-67$^+$/STn$^-$; and Ki-67$^+$/STn$^+$ areas.

mucins MUC1, MUC2 and MAUB (mucin antigen of the urinary bladder) isolated from bladder cancer cell lines carried STn (Bergeron et al., 1996, 1997). However, no evidence of such an expression was found in tumours. Herein, we readdressed this matter and found that the STn antigen was associated with advanced stage bladder tumours. More important, STn was absent in the healthy urothelium, which demonstrates its tumour-associated nature. Since this study was performed on a recent prospective series it is not possible, at this point, to determine correlations with disease outcome. Nevertheless, STn was mainly expressed by HG papillary NMIBC, known for their elevated risk of recurrence and progression to muscle invasive disease and MIBC that encompass an elevated risk of metastization and present decreased overall survival (Babjuk et al., 2012). STn expression was further associated with elevated Ki-67, a proliferation-related molecule and a surrogate biomarker of increased risk to recurrence and progression in bladder tumours (Margulis et al., 2009; Santos et al., 2003). In addition, the majority of non-proliferative tumours did not express STn, which demonstrates that the expression of the antigen is indeed a characteristic of proliferative tumours. Still, STn was mainly detected in non-proliferative areas of the tumours. However, the STn antigen was frequently observed in areas of invasion of the basal and muscle layers, suggesting it may be associated with the process of cell migration and invasion. This reinforces the notion that STn is part of a malignant bladder cancer phenotype, as previously observed for other carcinomas (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007). We also found the STn antigen in tumour-adjacent mucosa, which may be explained by the migration of STn$^+$ cells to the tumour surroundings. On the other hand, this may be a consequence of field carcinogenesis previously observed in bladder cancers (Jones et al., 2005; Palmeira et al., 2011). Nevertheless, the STn antigen holds potential as a biomarker of bladder disseminated disease.

STn is a product of an incomplete *O*-glycosylation process due to the premature *O*-6 sialylation of the glycoside GalNAcα1-*O*-Ser/Thr (Tn antigen) by ST6GalNAc.I (Marcos et al., 2004). In several epithelial tumours STn results from an increased ST6GalNAc.I expression and/or activity (Marcos et al., 2011; Sewell et al., 2006; Vazquez-Martin et al., 2004). Previous studies have reported ST6GalNAc.I expression by the urothelium at the mRNA level (Yamamoto et al., 2003); however we and others (Langkilde et al., 1992) have not detected STn expression in the histologically healthy tissues. These observations suggest either the absence of the antigen or the insufficient sensitivity of the method. ST6GalNAc.I localization in the Golgi apparatus and the competitive action of other glycosyltransferases for the Tn antigen may also favour the extension of

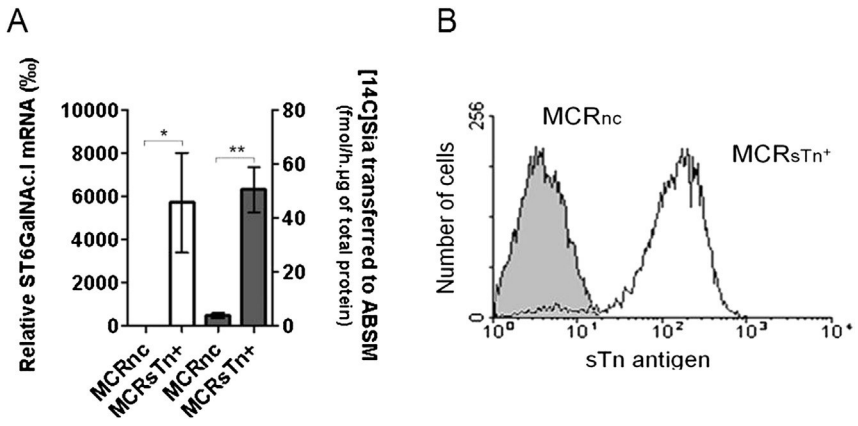

Figure 6 – ST6GalNAc.I mRNA expression and sialyltransferase activity in bladder cancer MCR cell lines. A) ST6GalNAc.I expression and activity in MCR cell lines. The relative mRNA levels of ST6GalNAc.I (open bars) and sialyltransferase activity towards ABSM (grey bars) were analysed as described in the Material and methods section. Both, ST6GalNAc.I mRNA and sialyltransferase activity towards ABSM are negligible in negative control cells and markedly increased upon ST6GalNAc.I transduction. B) Flow cytometry analyses of transduced MCR cells. Both negative control (MCRnc in grey histogram) and ST6GalNAc.I-transduced (MCRSTn$^+$ in open histogram) cell lines were stained with the secondary antibody anti-Ig's-FITC following incubation with the primary antibody anti-STn antigen. 90% of the ST6GalNAc.I-transduced cells expressed the STn antigen (MFI = 216). The data are shown as a mean ± standard deviation of 3 independent studies. "*" $p < 0.05$, "" $p < 0.01$ (Student's *T*-test).**

the O-glycan chain in non-pathological conditions. On the other hand we showed that the levels of STn in bladder tumours were correlated with the expression of ST6GalNAc.I, supporting this as a major molecular mechanism underlying STn biosynthesis in these tumours. Few cases presented STn expression associated with a basal level of ST6GalNAC.I, meaning that other factors may contribute to promote the biosynthesis of STn. A disorganization of secretory organelles (Sewell et al., 2006), somatic mutations in the gene *Cosmc*, encoding a molecular chaperone essential for O-chain elongation (Ju et al., 2008), the down-regulation/decreased activity of several other glycosyltransferases and/or the availability of sugar donors for biosynthesis, may also lead to STn overexpression. The integrated study of metabolic pathways, glycosyltransferases expression/activity, intra-cellular ultrastructures and microenvironmental changes may further enlighten the molecular events leading to abnormal O-glycosylation of bladder cancer proteins.

In addition we have screened HT1376, 5637, T24 and MCR bladder cancer cell lines and found neglectable levels of the STn antigen (data not shown). The same was previously observed in gastric (Ozaki et al., 2012; Pinho et al., 2007) and breast (Clement et al., 2004; Julien et al., 2005, 2006) cancers cell models, demonstrating that tumour cells may lose the ability to express this antigen *in vitro*. Microenvironmental factors may play a determinant role in the induction of STn biosynthesis, yet these events remain unknown. Following the association of STn with invasive cases, we elected the invasive bladder cancer cell line MCR to evaluate the biological role of STn in these tumours. We started by stably transducing the MCR cells with ST6GalNAc.I, which resulted in the overexpression of STn. The expression of STn did not promote a significant enhancement of MCR cell proliferation, which is agreement with observations made for breast (Clement et al., 2004; Julien et al., 2005, 2006) and gastric cancer models (Pinho et al., 2007). These findings associated with the absence of the antigen from most bladder tumours non-proliferative areas strongly suggests that STn expression does not play a direct role in tumour proliferation.

On the other hand, STn expression significantly enhanced the migration and invasive capacity of MCR cells, demonstrating that this antigen plays an important role in bladder cancer cell invasion, as suggested by the observation of bladder tumours. Enhanced migration capabilities of STn$^+$ cells on components of the extracellular matrix, such as fibronectin and collagen, have been described for other cancer cell lines (Julien et al., 2005, 2006; Pinho et al., 2007), and result, among several factors, from impaired integrin binding (Clement et al., 2004). In addition, STn expression has been shown to increase the invasion potential of tumour cells (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007), supporting a similar role in bladder tumours. Further experiments are however required to clarify the molecular mechanisms underlying promotion of cancer cell invasion and migration. These findings reinforce however that alterations in the glycosylation patterns of cell-surface proteins may strongly interfere with events like cell–cell adhesion, cell–matrix interaction, tumour growth, motility and invasion (Dall'Olio et al., 2012).

In resume, our work comprehensively describes the expression of the STn antigen in bladder cancer. Namely, it demonstrates the tumour-specific nature of this type of glycosylation and its association with advanced, highly proliferative tumours, invasion and organ disseminated disease. Thus, the evaluation of STn antigen may add valuable information about the aggressiveness of proliferative tumours, complementing the information given by Ki-67. Studies are ongoing in broader retrospective series to determine the association of STn with disease outcome and corroborate these findings. We are also devoted to the identification of the glycoproteins yielding STn, which is expected to bring insights

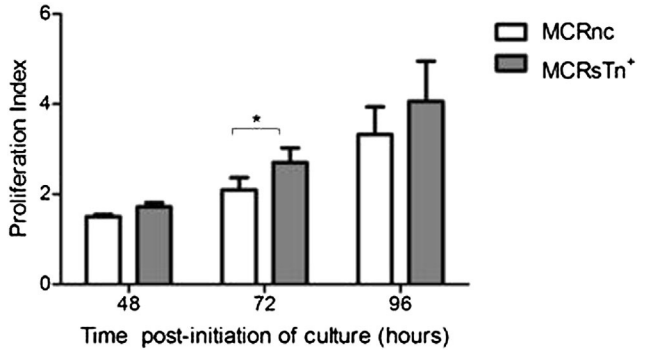

Figure 7 – Comparison between the proliferation capacity of MCRnc and MCRSTn$^+$ cells. The transduced MCR cells were labelled with CFSE and cultured for various periods of time (48, 72 and 96 h). The cells were harvested and analysed by flow cytometry with Modfit software, allowing the calculation of the proliferation index, which represents the average number of cells that was originated by a single cell of the parent generation. At the various periods of culture, MCRSTn$^+$ cells show a higher proliferation index than the negative control, but this difference was only statistically significant at 72 h of culture. The data are presented as a mean ± standard deviation of 3 independent studies. "*" $p < 0.05$ (Student's *T*-test).

about the role of this type of glycosylation in bladder carcinogenesis and provide novel therapeutic vectors. The antigen STn may also be monitored noninvasively in urine or serum using as is the case for other human carcinomas using the CA72-4 test (Reis et al., 2010). This could allow decreasing the number of cystectomies in post-surgery follow-ups of patients with high-grade tumours, a particularly critical matter for the elderly that constitute the majority of the cases.

Furthermore, the STn antigen is associated to high-grade NMIBC which currently constitutes one of the main therapeutics concerns due to their elevated risk of recurrence/progression (Babjuk et al., 2012). Adjuvant immunotherapy with BCG has allowed to delay recurrence and decrease the risk of progression into muscle invasive disease (Babjuk et al., 2012); still more than half of the patients either recur within two-years after TUR of the tumour or show intolerance to the treatment (Askeland et al., 2012; Yates and Roupret, 2011). Due to the lack of efficient therapies, upon therapeutic failure and/or muscle invasion, the patient is faced with cystectomy (Babjuk et al., 2012).

Carbohydrate antigens associated with advanced-stage tumours and malignant phenotypes such as STn, are expressed at the cell surface and, therefore, available for antibody or lectin-mediated recognition (Neutsch et al., 2012). Thus, these antigens may present an opportunity for the introduction of novel therapeutics, such as selective drug-delivery approaches (Neutsch et al., 2012) or carbohydrate-based immunotherapy (Heimburg-Molinaro et al., 2011). An anti-cancer vaccine named Theratope, comprehending a synthetic STn coupled to the immunogenic carrier keyhole limpet haemocyanin has already been developed (Julien et al., 2009; Miles et al., 2011; Sandmaier et al., 1999). Tests in animal models and humans for breast, ovarian, and colorectal cancers have showed that the antigen is safe and produces a strong immune response against these tumours (Julien et al., 2009, 2012; Miles et al., 2011). Even though Theratope failed to improve overall survival of metastatic breast cancer patients in a phase III clinical study, the design of the study disregarded the heterogeneous STn expression between patients (Miles et al., 2011), compromising the outcome (Julien et al., 2012; Zeichner, 2012). Thus, Theratope or other STn-based vaccine designs may constitute valuable therapeutic options for STn positive advanced bladder tumours. However, given the low association of STn with more proliferative areas of the tumour, one is led to speculate that advanced stage bladder cancer patients may better benefit from the combination of anti-STn immunotherapy and antiproliferative drugs. Furthermore, these approaches may allow targeting disseminated disease in the adjacent and distant mucosa from the main tumour.

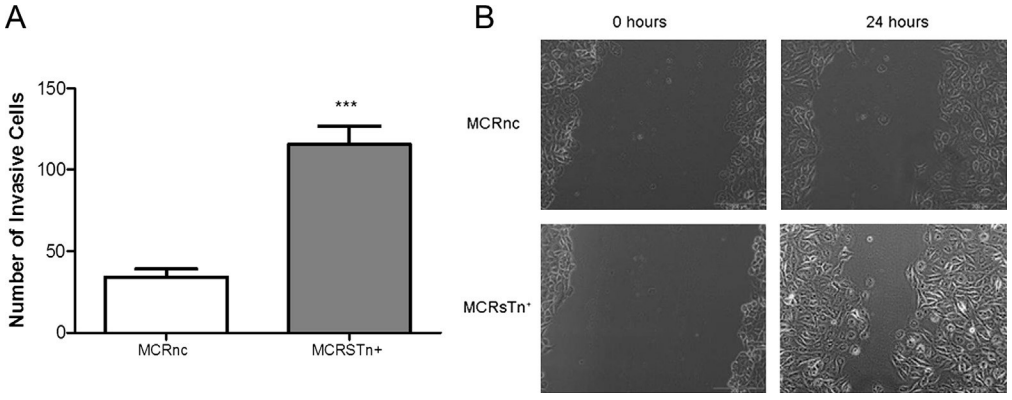

Figure 8 – STn expression promotes MCR cells wound healing closure and invasion. A) Wound healing closure assay. Uniform scratches were made using a 200 μL pipette tip in confluent monolayers of MCRSTn$^+$ and MCRnc cells. Cells were allowed to heal and the extent of closure was monitored by microscopic analysis. After 24 h culture, the MCRSTn$^+$ cells had almost completely covered the wound, in clear contrast to negative control, MCRnc, where unoccupied space was still observed. B) Invasion assay. MCRSTn$^+$ and MCRnc cells were incubated for 24 h, in the upper compartment of Matrigel invasion chambers, in complete DMEM medium and in the absence of other chemoattractants. Invasive cells were determined as described in Materials and methods. The data are presented as a mean ± standard deviation of 4 independent studies. "*" $p < 0.001$ (Student's *T*-test).

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3.2- Sialyl Tn and response to BCG immunotherapy

Paper II

Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy

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Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy

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Background: High risk of recurrence/progression bladder tumours is treated with Bacillus Calmette-Guérin (BCG) immunotherapy after complete resection of the tumour. Approximately 75% of these tumours express the uncommon carbohydrate antigen sialyl-Tn (Tn), a surrogate biomarker of tumour aggressiveness. Such changes in the glycosylation of cell-surface proteins influence tumour microenvironment and immune responses that may modulate treatment outcome and the course of disease. The aim of this work is to determine the efficiency of BCG immunotherapy against tumours expressing sTn and sTn-related antigen sialyl-6-T (s6T).

Methods: In a retrospective design, 94 tumours from patients treated with BCG were screened for sTn and s6T expression. *In vitro* studies were conducted to determine the interaction of BCG with high-grade bladder cancer cell line overexpressing sTn.

Results: From the 94 cases evaluated, 36 had recurrence after BCG treatment (38.3%). Treatment outcome was influenced by age over 65 years (HR = 2.668; (1.344–5.254); $P = 0.005$), maintenance schedule (HR = 0.480; (0.246–0.936); $P = 0.031$) and multifocallity (HR = 2.065; (1.033–4.126); $P = 0.040$). sTn or s6T expression was associated with BCG response ($P = 0.024$; $P < 0.0001$) and with increased recurrence-free survival ($P = 0.001$). Multivariate analyses showed that sTn and/or s6T were independent predictive markers of recurrence after BCG immunotherapy (HR = 0.296; (0.148–0.594); $P = 0.001$). *In vitro* studies demonstrated higher adhesion and internalisation of the bacillus to cells expressing sTn, promoting cell death.

Conclusion: s6T is described for the first time in bladder tumours. Our data strongly suggest that BCG immunotherapy is efficient against sTn- and s6T-positive tumours. Furthermore, sTn and s6T expression are independent predictive markers of BCG treatment response and may be useful in the identification of patients who could benefit more from this immunotherapy.

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Bladder cancer is the fifth most common cancer in Western society (van Rhijn *et al*, 2009), with the highest recurrence rate among solid tumours and poor prognosis when the tumour invades the muscularis propia (Babjuk *et al*, 2011). To reduce the risk of recurrence and progression to muscle invasion, non-muscle invasive high-grade tumours, multifocal and recurrent lesions are submitted to intravesical instillations with live attenuated Bacillus Calmette-Guérin (BCG) after complete transurethral resection of bladder tumour (TURBT; Babjuk *et al*, 2011). Although the management of the disease has significantly improved with this therapeutics, 30–40% of the patients relapse and approximately 15% progress to muscle invasive tumours (Palou Redorta, 2006). Predicting patients who could be best served by an alternative treatment or early cystectomy, would avoid progression, reduce disease charge and decrease health expenses. It is consensual that the integration of multiple biomarkers may hold predictive value; still such biomarker panel remains to be established (Lima *et al*, 2012; Zuiverloon *et al*, 2012).

The exact mechanism by which BCG mediates anti-bladder cancer immunity remains elusive (Alexandroff *et al*, 2010). However, the capability of the bacillus to recognise and efficiently bind to tumour cells has a determinant role in the therapeutics outcome (Alexandroff *et al*, 2010). The bacillus is then internalised triggering tumour cell apoptosis or host adaptive immune responses (Becich *et al*, 1991; Ratliff, 1992). The bacterial adhesion, fibronectin attachment protein (FAP), was recognised as the main factor mediating BCG attachment and internalisation by bladder tumour cells (Sinn *et al*, 2008; Alexandroff *et al*, 2010). FAP binds to $\alpha5\beta1$ integrins expressed by tumour cells via a fibronectin bridge and to be responsible for the uptake of BCG–fibronectin–integrin complexes (Sinn *et al*, 2008; Alexandroff *et al*, 2010).

Malignant transformations may be accompanied by a premature stop in the *O*-glycosylation of proteins by sialylation, originating the sialyl-Tn (sTn, Neu5Acα2-6GalNAcα-*O*-Ser/Thr) and sialyl-6-T antigens (s6T, Galβ1-3(Neu5Acα2-6)GalNAcα-O-Ser/Thr; Dall'Olio *et al*, 2012). We recently reported that approximately 75% of high-grade bladder tumours, presenting elevated proliferation indexes and high risk of recurrence/progression expressed sTn (Ferreira *et al*, 2013). sTn expression enhanced the invasive capability of bladder cancer cells and was considered a surrogate biomarker of tumour aggressiveness (Ferreira *et al*, 2013). Hence, efficient therapies to manage these tumours are needed to avoid disease progression and poor outcomes.

sTn expression is known to interfere with cell–cell adhesion, cell–matrix interaction, including integrin-fibronectin binding, modulate cell morphology (Clement *et al*, 2004; Julien *et al*, 2006; Pinho *et al*, 2007) and immune responses (Gilewski *et al*, 2007; Julien *et al*, 2009; Takamiya *et al*, 2013). Thus, we hypothesise it may modulate BCG attachment to tumour cells and/or immune response and consequently influence BCG immunotherapy outcome. sTn is also a biomarker of concomitant molecular alterations that may further determine the tumour behaviour (Ohno *et al*, 2006). As such, this work is devoted to evaluating the response of sTn-positive bladder tumours to BCG immunotherapy. The sTn structurally related antigen was also evaluated for the first time in the context of bladder cancer.

MATERIALS AND METHODS

Patient cohort. This study was performed in a retrospective series of 94 cases with high-risk non-muscle invasive bladder cancer. Patients were treated with TURBT and then submitted to BCG immunotherapy in the Portuguese Oncology Institute of Porto, between 1998 and 2006. No second-look TURBT was performed, although the majority of the samples had muscularis propria tumour free. All received intravesical instillation of BCG for 6 consecutive weeks (induction BCG scheme, iBCG) and 56.4% were submitted to maintenance BCG schedule (iBCG + maintenance protocol with two weekly instillations every 3 months during 2 years, mBCG). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG (Oosterlinck *et al*, 2006) scheme and patients showing significant intolerance to long BCG treatment.

The male/female sex ratio was of 78:16. The patients were followed every 3 months for the first year, every 6 months for the second year and every 12 months thereafter by cystoscopy and urine cytology. Recurrence was defined as the appearance of a tumour once the treatment has begun, with at least one tumour-free cystoscopy and cytology in-between. These recurrences were also available for study. The non-responders were defined as patients submitted to BCG treatment with tumour recurrence. Finally, recurrence-free survival (RFS) was defined as the period of time between the beginning of treatment and recurrence or the most recent tumour-free cystoscopy and cytology. All procedures were performed after patient's informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients' clinical records. All tumour samples were revised by a pathologist, regarding 2004 WHO grading criteria.

Expression of STn in bladder tumours. Formalin-fixed paraffin-embedded tissue sections were screened for sTn by immunohistochemistry using the avidin/biotin peroxidase method, as described by Ferreira *et al* (2013). sTn expression was evaluated with anti-sTn mouse monoclonal antibody clone TKH2 (Ferreira *et al*, 2013). The s6T antigen was evaluated in sTn-negative tumours using the same antibody, after treatment with a recombinant β-(1-3)-galactosidase from *Xanthomonas campestris* (R&D systems, Minneapollis, MN, USA) for 1 h at 37° C. This enzyme removes the *O*-3-linked Gal residues exposing the sTn antigen (Figure 1A).

Both antigens were assessed double-blindly by three independent observers. Upon disagreement, the slides were reviewed, until a consensus was reached. Tumours were classified as positive when immunoreactivity of anti-sTn TKH2 antibody was observed.

Structural assignments were validated by a combination of enzymatic treatments. For sTn, positive tissues were first treated with a α-neuraminidase from *Clostridium perfringens* (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37 °C to remove the sialic acid and then screened for sTn expression. For s6T, positive tissues were primarily incubated with the β-(1–3)-galactosidase, followed by incubation with the neuraminidase. The absence or decrease in immunoreactivity of TKH2 monoclonal antibody confirmed the presence of these structures.

Adhesion and internalisation of BCG to bladder cancer cell line

Cell lines culture. The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and MCRsTn$^+$) were grown as described by Videira *et al* (2009). The MCRsTn$^+$ cell line results from the stable transduction of MCR cells with a lentivirus expressing the coding region of the human *ST6GalNAc.I* gene, the enzyme responsible by the biosynthesis of sTn (Ferreira *et al*, 2013). The MCRnc cell line, not expressing sTn, was used as control (Ferreira *et al*, 2013).

Bacterial strain and labelling. BCG strain RIVM (Medac, Hamburg, Germany) was used in this study. Briefly, 10^8 viable BCG cells were labelled with 10 μg fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, CA, USA) in 1 ml of 50 mM sodium carbonate buffer (pH 9.2) for 30 min at 20 °C. The labelled bacteria (BCG-FITC) were washed three times with PBS containing 0.05% of Tween-80 (Sigma-Aldrich) and centrifuged for 10 min at 13.000 g to remove excess FITC.

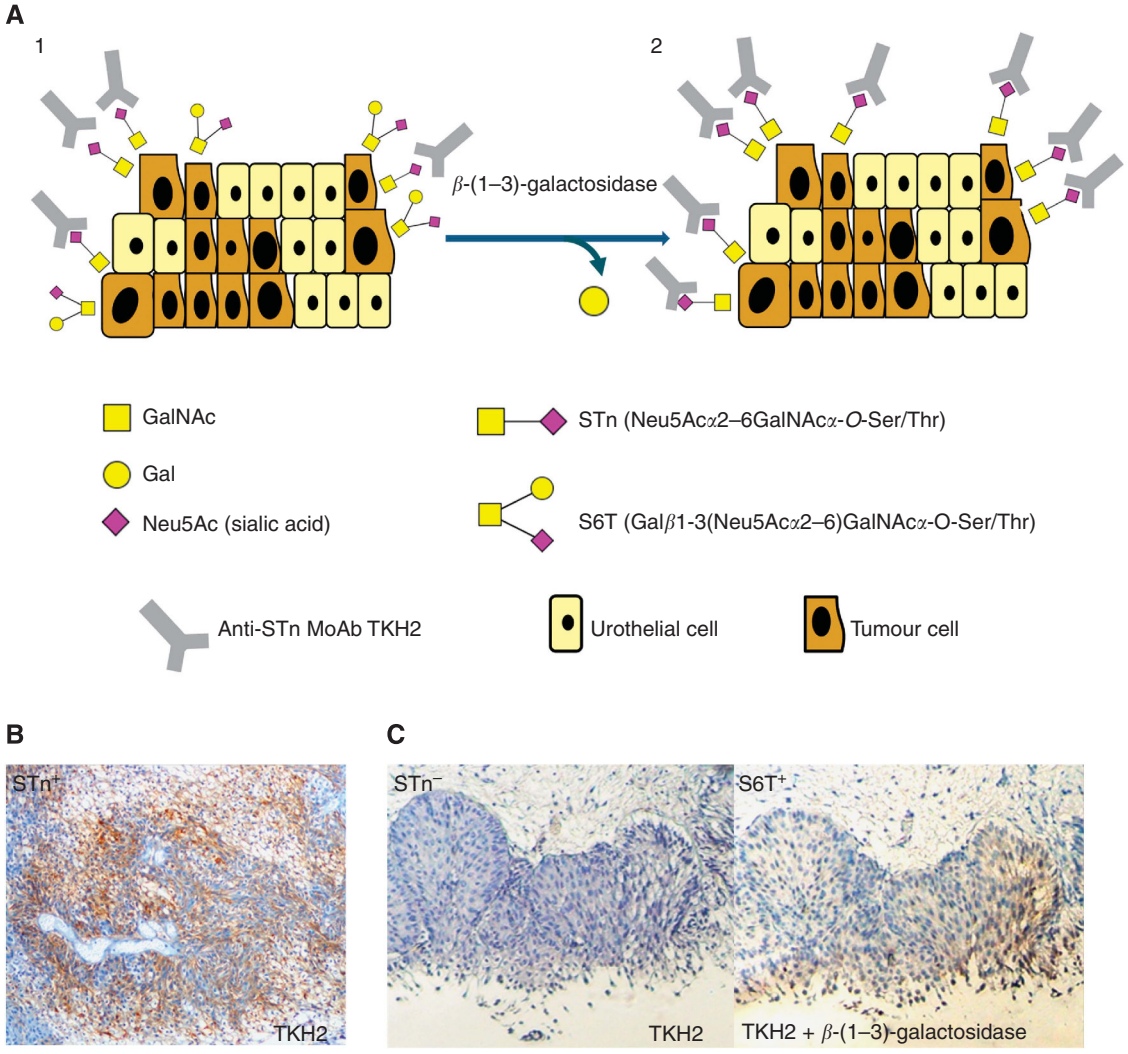

Figure 1. Expression of sTn and s6T in bladder tumours. Schematic representation of a bladder tumour expressing sTn and s6T antigens (**A**). Urothelial cell-surface glycoproteins present more or less elongated *O*-glycan chains often terminated with ABO and/or Lewis blood group determinants (not represented). Conversely, some malignant cells express the sTn and s6T (STn + Gal residue) antigens that result from a premature stop in the biosynthesis of *O*-glycans (**A**). The sTn, but not the s6T antigen, can be recognised by mouse monoclonal antibody TKH2 (**A**1). The digestion of the FFPE sections with a *β*-(1-3)-galactosidase removes the Gal residue from s6T allowing detection by TKH2 (**A**2). Bladder tumour expressing the sTn antigen (**B**). Bladder tumour expressing the s6T antigen but not sTn (**C**). The FFPE tissue was positive for sTn only after digestion of the FFPE tissue with a *β*-(1-3)-galactosidase. This is the first report on s6T antigen expression in bladder tumours.

BCG adhesion and internalisation assay. MCR cells were plated into 24-well plates at 0.2×10^6 cell per well. At confluence, cells were washed and BCG-FTIC was added to the cultures at a ratio of 10:1 (BCG/cells). After 2, 6 or 24 h incubation time, the cells were gently washed to remove the excess BCG, harvested and centrifuged at 350 **g** for 5 min. In order to differentiate between internalised and surface-bound BCG, trypan blue was added to quench surface-attached fluorescence bacteria. Flow cytometry was performed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and data were analysed using the Flowing *v*2.4 software (Turku Center for Biotechnology, Turku, Finland).

Estimation of cell viability after exposure to BCG. The influence of BCG treatment on MCR cell viability after the 24 h of exposure was assessed through the visualisation of morphologic changes by flow cytometry. Dot plots of forward-angle light scatter (FSc) *vs* side-angle light scatter (SSc) of MCR cells before and after exposure to BCG were analysed using the Flowing *v*2.4 software (Turku Center for Biotechnology).

For a shorter period of time of BCG exposure (6 h), apoptotic status of cells was evaluated by labelling with Annexin V (Sigma-Aldrich) as indicated by manufacture instructions.

Statistical analysis. Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0; IBM, Armonk, NY, USA). Chi-square analysis was used to compare categorical variables. Kaplan–Meier survival curves were used to evaluate correlation between glycans expression and RFS, log-rank statistical test was used for curves comparison. Multiple Cox regression analysis was used to assess the effect of both antigens on the time to recurrence in BCG-treated patients and to adjust for potential confounders. Non-parametric Mann–Whitney test was used to compare the differences in the BCG attachment and internalisation to MCRnc and MCRsTn+ cells.

RESULTS

Clinicopathological features and BCG treatment outcome. From the 94 cases evaluated, 36 had recurrence after BCG

Table 1. Relation between patients clinical and tumour characteristics and response to BCG treatment and time to recurrence

Variables	Total *n* (%)	Responders *n* (%)	Non-responders *n* (%)	*P*[a]	HR (95% CI)	*P*[b]
Age (years)						
<65	51 (54.3)	37 (63.8)	14 (38.9)	**0.018**	1.0	
⩾65	43 (45.7)	21 (36.2)	22 (61.1)		2.668 (1.355–5.254)	**0.005**
Sex						
Male	78 (83.0)	47 (81.0)	31 (86.1)	0.524	1.0	
Female	16 (17.0)	11 (19.0)	5 (13.9)		0.883 (0.342–2.283)	0.798
Stage						
Ta	40 (42.6)	23 (39.7)	17 (47.2)	0.471	1.0	
T1	54 (57.4)	35 (60.3)	19 (52.8)		0.838 (0.435–1.613)	0.596
Grade						
Low	38 (40.4)	24 (41.4)	14 (38.9)	0.843	1.0	
High	56 (59.6)	34 (58.6)	22 (61.1)		1.295 (0.661–2.537)	0.450
Size (cm)						
<3	62 (66.7)	38 (65.5)	24 (68.6)	0.762	1.0	
⩾3	31(33.3)	20 (34.5)	11 (31.4)		0.787 (0.384–1.613)	0.513
Tumour number						
Unifocal	51 (54.3)	30 (51.7)	13 (36.1)	0.140	1.0	
Multifocal	43 (45.7)	28 (48.3)	23 (63.9)		2.065 (1.033–4.126)	**0.040**
CIS						
No	88 (93.6)	54 (93.1)	34 (94.4)	1.000	1.0	
Yes	6 (6.4)	4 (6.9)	2 (5.6)		0.783 (0.188–3.267)	0.737
Recurrence status						
Primary	48 (51.0)	31 (53.4)	17 (47.2)	0.557	1.0	
Recurrent	46 (49)	27 (46.6)	19 (52.8)		1.327 (0.686–2.564)	0.401
BCG schedule						
iBCG	41 (43.6)	20 (34.5)	21 (58.3)	**0.023**	1.0	
mBCG	53 (56.4)	38 (65.5)	15 (41.7)		0480 (0.246–0.936)	**0.031**

Abbreviations: BCG = Bacillus Calmette-Guérin; CI = confidence interval; CIS = Carcinoma *in situ*; HR = hazard ratio; iBCG = induction BCG; mBCG = maintenance BCG.
Bold values indicate $P<0.05$.
[a]Chi-square test.
[b]Wald test.

treatment (38.3%). The median follow-up time of the patients free of recurrence was 68.5 months (range: 6.0–135.0) and the median time of recurrence was 38.5 months (range: 10.0–122.0). The median follow-up time considering all the cases under analysis was 61.0 months (range: 6.0–135.0). Table 1 summarises patients and tumour clinicopathological features and its association with BCG response and RFS after treatment. We found that 61.1% of the non-responders were over 65 years old at the time of tumour resection, whereas only 36.2% of responders were over 65 years old ($P=0.018$). Furthermore, patients over 65 years have approximately three-fold increased risk of recurrence (HR = 2.668; (1.344–5.254); $P=0.005$). Moreover, it was observed a higher percentage of patients treated only with iBCG in the non-responder group when compared with the responder group (58.3% *vs* 34.5%, $P=0.018$). Therefore, patients treated with mBCG scheme showed a 52% reduced risk of recurrence (HR = 0.480; (0.246–0.936); $P=0.031$). It was also found that patients with multifocal tumours had an increased risk of recurrence after BCG treatment (HR = 2.065; (1.033–4.126); $P=0.040$). No association was found for other characteristics, such as gender, tumour stage, grade or size, CIS presence and prior recurrence.

Expression of sTn and s6T and association with clinicopathological features. Approximately 66% of the studied bladder tumours were sTn positive (Figure 1A and B) and in all cases the antigen was observed in more than 5% of the tumour area. Additionally, 10 out of 32 sTn negative cases were positive for s6T (Figure 1C), which is structurally related to sTn. However, s6T assumed a more diffuse expression that did not exceed 5% of the tumour area in all cases.

The expression of sTn alone or in combination with s6T (sTn/s6T) was associated with high-grade tumours ($P=0.007$; $P=0.037$ Table 2) and also with primary tumours ($P=0.001$; $P=0.039$).

STn and sTn/s6T as predictors of BCG treatment outcome. sTn antigen was expressed by 74.1% of BCG responders and only by 47.2% of non-responders ($P=0.034$; Table 3). When sTn and s6T were evaluated together, a similar relationship was observed ($P=0.0001$; Table 3).

From the 94 patients included in this study, 36 had recurrences after treatment and 75% of these tumour specimens were available for sTn and s6T screening. All non-responders who presented sTn-

Table 2. Association between sTn and s6T antigens and clinicopathological characteristics

	sTn			sTn + s6T		
Variables	**Negative *n* (%)**	**Positive *n* (%)**	***P*[a]**	**Negative *n* (%)**	**Positive *n* (%)**	***P*[a]**
Age (years)						
<65	21 (65.6)	30 (48.4)	0.112	13 (59.1)	38 (52.8)	0.603
⩾65	11 (34.4)	32 (51.6)		9 (40.9)	34 (47.2)	
Sex						
Male	27 (84.4)	51 (86.1)	0.796	17 (77.3)	61 (84.7)	0.517
Female	5 (15.6)	11 (17.7)		5 (22.7)	11 (15.3)	
Stage						
Ta	16 (50.0)	24 (38.7)	0.294	10 (45.5)	30 (41.7)	0.753
T1	16 (50.0)	38 (61.3)		12 (54.5)	42 (58.3)	
Grade						
Low	19 (59.4)	19 (30.6)	**0.007**	13 (59.1)	25 (34.7)	**0.042**
High	13 (40.6)	43 (69.4)		9 (40.9)	47 (65.3)	
Size (cm)						
<3	23 (74.2)	39 (62.9)	0.276	16 (76.2)	46 (63.9)	0.431
⩾3	8 (25.8)	23 (37.1)		5 (23.8)	26 (36.1)	
Tumour number						
Unifocal	12 (37.5)	13 (50.0)	0.249	7 (31.8)	36 (50.0)	0.134
Multifocal	20 (62.5)	31 (50.0)		15 (68.2)	36 (50.0)	
CIS						
No	29 (90.6)	59 (95.2)	0.406	19 (86.4)	69 (95.4)	0.112
Yes	3 (9.4)	3 (4.8)		3 (13.6)	3 (4.2)	
Recurrence status						
Primary	9 (28.1)	39 (62.9)	**0.001**	7 (31.8)	41 (56.9)	**0.039**
Recurrent	23 (71.9)	19 (37.1)		15 (68.2)	31 (43.1)	
BCG schedule						
iBCG	14 (43.8)	27 (43.5)	0.985	11 (50.0)	30 (41.7)	0.490
mBCG	18 (56.2)	35 (56.5)		11 (50.0)	42 (58.3)	

Abbreviations: BCG = Bacillus Calmette-Guérin; iBCG = induction BCG; CIS = Carcinoma *in situ*; mBCG = maintenance BCG.
Bold values indicate $P<0.05$.
[a]Chi-square test.

Table 3. STn and sTn/s6T frequencies and risk of recurrence after BCG therapy

	Responders *n* (%)	**Non-responders *n* (%)**	***P* value[a]**
sTn			
Negative	15 (25.9)	17 (47.2)	
Positive	43 (74.1)	19 (52.8)	0.034
sTn and/or s6T			
Negative	6 (10.5)	16 (43.2)	
Positive	51 (89.5)	21 (56.8)	0.0001

Abbreviation: BCG = Bacillus Calmette-Guérin.
[a]Chi-square test.

negative tumours prior treatment had sTn-negative recurrences. From the 15 non-responder patients who had sTn-positive tumours, 40% presented sTn-negative recurrences.

Kaplan–Meier analysis was used to evaluate if sTn with or without s6T influenced the RFS after BCG treatment. No differences were found regarding sTn expression alone (Figure 2A). However, when sTn and s6T were considered together, significant differences were found ($P=0.001$; Figure 2B). Patients with sTn/s6T-positive tumours had higher RFS than negative tumours (100.1 *vs* 63.2 months).

A Cox regression analysis, adjusted to age, tumour number and treatment scheme, was performed to assess the individual effect of these antigens in recurrence after BCG. Patients with sTn-positive tumours presented a trend to a lower-risk recurrence after BCG (HR = 0.544; 95% CI: (0.275–1.076); $P=0.080$; Table 4). Likewise, cases positive for sTn/S6T showed a significant lower risk of recurrence (HR = 0.296; 95% CI: (0.148–0.594); $P=0.001$).

BCG interaction with MCRsTn$^+$ cell line. To evaluate the affinity of BCG for cells expressing sTn, we set up *in vitro* assays with MCRnc and MCRsTn$^+$ cell lines, two genetically modified variants of the original MCR bladder cancer cell line. MCRnc cells

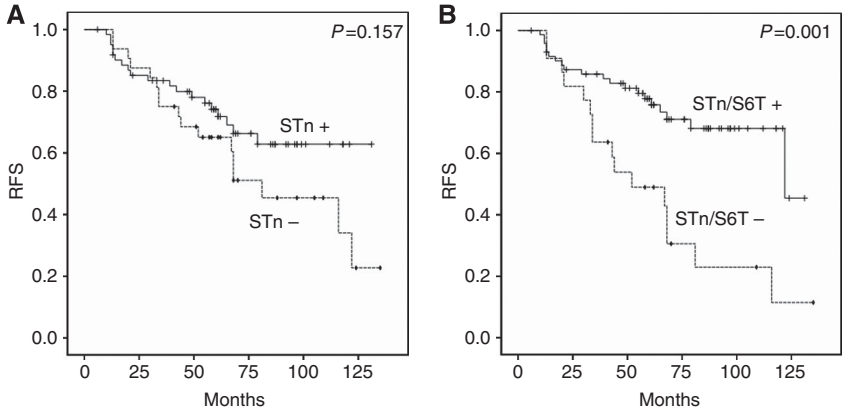

Figure 2. Effect of sTn and s6T expression in recurrence-free survival (RFS). Kaplan–Meier analysis to evaluate the association between RFS in the studied patients and: (**A**) sTn expression; (**B**) STn plus s6T presence (sTn/S6T). Comparison performed by log-rank test (**A**: $P=0.157$; **B**: $P=0.001$); ± censored sTn or sTn/s6T-negative tumours; ♦ censored sTn or sTn/s6T-negative tumours.

Table 4. Multivariate analysis and risk estimation of sTn and s6T influence on BCG therapy outcome

	HR[a]	95% CI	*P* value
sTn			
Negative	1.0	Referent	
Positive	0544	0275–1.076	0.080
sTn + s6T			
Negative	1.0	Referent	
Positive	0296	0148–0.594	0.001

Abbreviations: BCG = Bacillus Calmette-Guérin; CI = confidence interval; HR = hazard ratio.
[a]Adjusted for age, tumour number and BCG schedule.

were transduced with an empty vector and MCRsTn$^+$ with the cDNA coding for the ST6GalNAc.I sialyltransferase. The phenotype of these transduced variants was previously described by Ferreira *et al* (2013). MCRsTn$^+$ presents a continuous and stable expression of the antigen sTn, whereas the MCRnc does not as shown in Supplementary Figure 1 (in the supplementary section).

MCRsTn$^+$ and its negative control MCRnc where then treated with BCG. Fluorescent-labelled BCG was internalised significantly over time, with a significant higher internalisation after 6 h incubation, when comparing with 2 h incubation (Figure 3A). Interestingly, after 6 h, MCRsTn$^+$ cells showed a tendency to internalise higher amounts of BCG than MCRnc cells (Figure 4A and B). Small amounts of BCG are internalised, resulting in small shifts of FITC fluorescence of the cells after internalisation (Figure 4B).

We then evaluated apoptosis status in MCR cells after 6 h of BCG challenging, using Annexin V an earlier labelling marker for cells undergoing apoptosis. It was observed a consistent tendency for a higher cell death in MCRsTn$^+$ after BCG challenging (higher Annexin V labelling—$MFI_{MCRnc}=2560$) compared with MCRnc cells ($MFI_{MCRsTn+}=2640$). In addition, a population of cells presenting stronger Annexin V labelling was also observed after 6 h BCG, which was higher (7%) in MCRsTn$^+$ cell than MCRnc (4%; Figure 4A). After a longer period of BCG challenging (24 h), MCRsTn$^+$ cells significantly decreased their size and granularity (80% of $FSC^{low}SSC^{low}$), which is usually typical of a rupture of plasma membrane and leakage of the cell's contents (Figure 4B). Conversely, MCRnc cells underwent little physical changes, presenting only 10% of $FSC^{low}SSC^{low}$ (Figure 4B). Furthermore, the internalisation of BCG by viable MCRsTn$^+$ cells at 24 h was markedly increased in relation to the controls (MCRnc; Supplementary Figure 2, supplementary section), therefore, in accordance with the observations made for 6 h.

These results present evidence that both BCG internalisation and loss of cell viability are correlated and both features are enhanced in cells expressing sTn antigen. Altogether, these findings suggest that the bacillus acts more efficiently in cells expressing sTn probably due to its higher internalisation.

DISCUSSION

A significant percentage of high risk of recurrence/progression bladder tumours, conservatively treated with BCG immunotherapy after surgery, express cell-proteins yielding the sTn antigen (Ferreira *et al*, 2013). Despite the malignant potential of these tumours (Ferreira *et al*, 2013) and evidences that sTn expression may modulate the cell–BCG interaction (Clement *et al*, 2004; Julien *et al*, 2006; Pinho *et al*, 2007) as well as immune responses (Gilewski *et al*, 2007; Julien *et al*, 2009; Takamiya *et al*, 2013), nothing is known about the way patients exhibiting sTn-positive tumours respond to treatment.

We first observed that treatment outcome was influenced by age, treatment scheme and tumour multifocallity, as showed in other reports (Bohle and Bock, 2004; Joudi *et al*, 2006; Fernandez-Gomez *et al*, 2008; Malmstrom *et al*, 2009; Kohjimoto *et al*, 2010; Ajili *et al*, 2012). To overcome the samples heterogeneity, these variables were taken into account in multivariate analysis models to assess the influence of tumour-associated glycans in BCG response.

We also found that sTn expression was associated with high-grade tumours, which is in agreement with our previous observations (Ferreira *et al*, 2013). STn expression was also associated with primary tumours. However, this may result from fact that the percentage of high-grade tumours was much higher among primary cases (90%) than in recurrences (67%). Altogether, these data reinforce the notion that sTn is a surrogate marker of high-risk bladder cancer.

Furthermore, we report for the first time that bladder tumours express the sTn-related carbohydrate antigen s6T. From the structural point of view, s6T may be considered a form of the sTn antigen masked by a Gal residue *O*-3 linked to the GalNAc moiety. To our knowledge, s6T has only been observed in human cancer cell lines (Marcos *et al*, 2004; Julien *et al*, 2006; Pinho *et al*, 2007). We also describe that the incubation of tissue

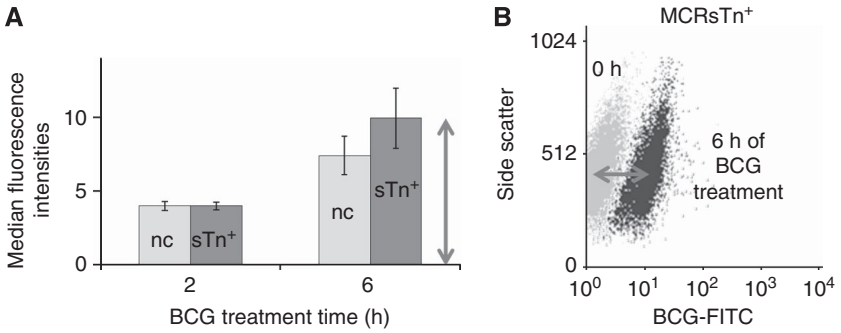

Figure 3. BCG internalisation by bladder cancer cell lines over time. (**A**) Internalisation of fluorescent-labelled BCG by mock-transduced MCRnc cells (light grey bars) and by *ST6GalNAc.I*-transduced MCRsTn$^+$ cells (dark grey bars), after 2 or 6 h of exposure with BCG. An obvious time-dependent internalisation was observed, and a tendency for higher fluorescent-labelled BCG internalisation rates was observed by MCRsTn$^+$ cells. Data are the average of three independent experiments (mean fluorescence intensity). (**B**) A representative flow cytometry dot plots of MCRsTn$^+$ cells before (light grey dots) and after 6 h (dark grey dots) of fluorescent-labelled BCG exposure. Horizontal and transversal double sense arrows represent the MFI shift observed after 6 h of BCG internalisation.

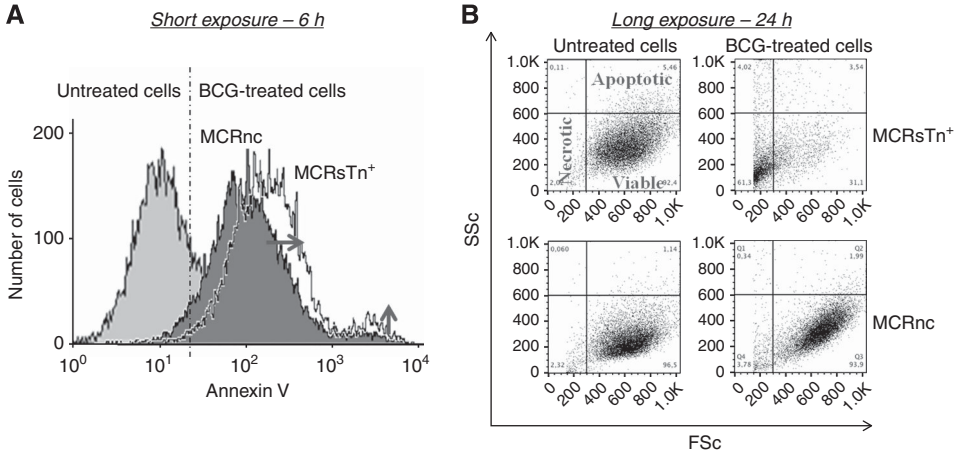

Figure 4. Effect of BCG in the viability of bladder cancer cell lines expressing sTn. (**A**) The apoptotic status of MCR cell lines was evaluated after 6 h exposure to BCG, by Annexin V staining and flow cytometry analysis. Flow cytometry histograms show MCRnc and MCRsTn$^+$ cells labelled with Annexin V before (light grey histograms) and after 6 h of BCG treatment (dark grey and open histograms, respectively). MCRsTn$^+$ cells showed higher Annexin V labelling ($MFI_{MCRnc} = 2560$ to $MFI_{MCRsTn+} = 2640$—horizontal arrow) and a higher percentage of cells with strong Annexin V labelling (4% to 7%—vertical arrow). (**B**) Analysis of size and granularity of cells exposed 24 h to BCG. Dot plot analysis of BCG-treated MCRsTn$^+$ revealed a marked decrease of both side-angle light scatter (SSc) and forward-angle light scatter (FSc) signals, which is consistent with massive cell death. By contrast, BCG treatment resulted in little changes of the dot plot pattern of MCRnc cells. Data are from one representative assay out of three independent experiments.

sections with a β-(1-3)-galactosidase removed the *O*-3-linked Gal residue exposing the sTn antigen, allowing its detection by immunohistochemistry with the same antibody used for sTn without significant time consumption. This approach may now be applied to estimate s6T expression in other solid tumours. Studies *in vitro* studies have shown that s6T expression influences tumour microenvironment similarly to sTn (Pinho *et al*, 2007). Therefore, both antigens were evaluated in the context of BCG immune response.

STn expression alone or in combination with that of s6T was associated with lower recurrence rates after BCG. Furthermore, patients expressing sTn and/or s6T presented longer RFS and these antigens were found to be independent predictive markers of reduced recurrence after BCG immunotherapy. Moreover, recurrences after treatment displayed a reduced expression of sTn antigens suggesting that BCG may be more effective against cells expressing these glycans. Thus, sTn-like *O*-glycans should be considered in a biomarker panel directed to predict BCG treatment outcome.

STn-expressing cells presented enhanced capacity for BCG adhesion and internalisation and higher BCG-mediated cell death *in vitro*. This strongly suggests that sTn expression favours BCG-mediated elimination of tumour cells, which may, in part, explain the high correlation between these glycans and treatment response. The exact mechanism underlying these observations remains unknown. However, BCG is known to bind fibronectin–$\alpha5\beta1$ integrin complexes promoting a rearrangement of cytoskeletal actin in host cells, which results in the phagocytosis of the pathogen (Chen *et al*, 2003; Alexandroff *et al*, 2010). Clement *et al* (2004) described that integrin $\beta1$ chains express sTn and that the antigen enhanced integrin-fibronectin adhesion. Thus, sTn may contribute to a more efficient binding of the bacillus to tumour cells and consequently a better response to BCG. The bacillus may also directly target cells in a fibronectin-independent manner (Schneider *et al*, 1994), namely by binding sTn or specific carbohydrates residues such as $\alpha2,6$ sialic acids. On the other hand, sTn is a product of incomplete *O*-glycosylation of proteins (Dall'Olio *et al*, 2012), a reduction in the structural complexity of

O-glycan may allow the bacillus to bind more efficiently to tumour cells. The expression of sTn also induces profound morphological changes in tumour cells (Clement *et al*, 2004; Pinho *et al*, 2007) that may further contribute to the bacillus attachment. A deeper understanding of these phenomena may provide new insights on the mechanism of action of BCG and ways to improve the therapeutics.

The efficiency of BCG therapy among sTn-positive tumours may also be related with the immunogenic properties of the antigen (Gilewski *et al*, 2007; Julien *et al*, 2009; Takamiya *et al*, 2013). sTn-based vaccines elicit strong immune responses against breast, ovarian and colorectal cancers in animal models (Gilewski *et al*, 2007; Julien *et al*, 2009). Still, using BCG as an immunologic adjuvant was considered essential for the development of both humoral and cellular immune responses against sTn (Miles *et al*, 1996; O'Boyle *et al*, 2006). Similarly, instillations with BCG may be enhancing immune responses against these tumour-associated glycans. On the other hand, we observed a higher internalisation of BCG by sTn-expressing cells. After BCG internalisation, malignant cells often act as antigen-presenting cells contributing to the immunologic cascade that leads to tumour clearance (Ratliff, 1992; Alexandroff *et al*, 2010). Therefore, one may also hypothesise that the expression of this particular glycan may increase the probability of generating BCG antigen-presenting cells in the tumour niche. Understanding these events may allow developing alternative carbohydrate-based immunotherapies for bladder cancer and should be addressed in future studies.

Although this is a retrospective study involving a limited number of cases, our results strongly suggest that BCG immunotherapy is efficient against sTn-positive tumours. Even though we have not determined the exact mechanisms underlying this event, we demonstrated that BCG adhesion and internalisation is higher for sTn-positive cells *in vitro*, further reinforcing tumour findings.

In conclusion, it has been demonstrated that sTn and s6T antigens correlated with a better response to this treatment. These glycans, in association with other BCG response-associated molecules, may allow the establishment of a predictive panel that can guide therapeutic decision.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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3.3- Sialyl Tn and its interference in cancer biological mechanisms

Paper III

Abnormal protein glycosylation and activated pi3k/akt/mtor pathway: role in bladder cancer prognosis and targeted therapeutics

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RESEARCH ARTICLE

Abnormal Protein Glycosylation and Activated PI3K/Akt/mTOR Pathway: Role in Bladder Cancer Prognosis and Targeted Therapeutics

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Abstract

Muscle invasive bladder cancer (MIBC, stage ≥T2) is generally associated with poor prognosis, constituting the second most common cause of death among genitourinary tumours. Due to high molecular heterogeneity significant variations in the natural history and disease outcome have been observed. This has also delayed the introduction of personalized therapeutics, making advanced stage bladder cancer almost an orphan disease in terms of treatment. Altered protein glycosylation translated by the expression of the sialyl-Tn antigen (STn) and its precursor Tn as well as the activation of the PI3K/Akt/mTOR pathway are cancer-associated events that may hold potential for patient stratification and guided therapy. Therefore, a retrospective design, 96 bladder tumours of different stages (Ta, T1-T4) was screened for STn and phosphorylated forms of Akt (pAkt), mTOR (pmTOR), S6 (pS6) and PTEN, related with the activation of the PI3K/Akt/mTOR pathway. In our series the expression of Tn was residual and was not linked to stage or outcome, while STn was statically higher in MIBC when compared to non-muscle invasive tumours ($p = 0.001$) and associated decreased cancer-specific survival (log rank $p = 0.024$). Conversely, PI3K/Akt/mTOR pathway intermediates showed an equal distribution between non-muscle invasive bladder cancer (NMIBC) and MIBC and did not associate with cancer-specif survival (CSS) in any of

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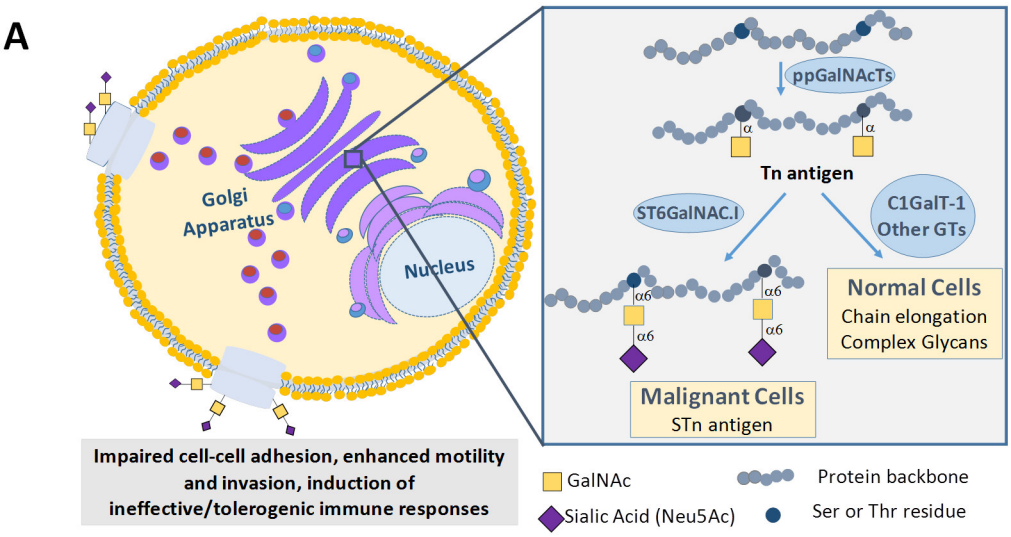
these groups. However, the overexpression of pAKT, pmTOR and/or pS6 allowed discriminating STn-positive advanced stage bladder tumours facing worst CSS (p = 0.027). Furthermore, multivariate Cox regression analysis revealed that overexpression of PI3K/Akt/mTOR pathway proteins in STn+ MIBC was independently associated with approximately 6-fold risk of death by cancer (p = 0.039). Mice bearing advanced stage chemically-induced bladder tumours mimicking the histological and molecular nature of human tumours were then administrated with mTOR-pathway inhibitor sirolimus (rapamycin). This decreased the number of invasive lesions and, concomitantly, the expression of STn and also pS6, the downstream effector of the PI3K/Akt/mTOR pathway. In conclusion, STn was found to be marker of poor prognosis in bladder cancer and, in combination with PI3K/Akt/mTOR pathway evaluation, holds potential to improve the stratification of stage disease. Animal experiments suggest that mTOR pathway inhibition could be a potential therapeutic approach for this specific subtype of MIBC.

Introduction

Bladder cancer is the second most deadly genitourinary tumour and presents significantly worse prognosis upon *muscularis propria* invasion [1]. Approximately 20–30% of the newly diagnosed cases are muscle invasive bladder cancers (MIBC; T2-T4 stages), while 50% are non-muscle invasive bladder tumours (NMIBC) with high potential to progress to invasion. MIBC treatment includes cystectomy and (neo)adjuvant cisplatin-based chemotherapy regimens [2]. However, significant variations in the natural history of the disease and responses to treatment can be observed between tumours with identical histological features, reflecting their high molecular heterogeneity [3]. Furthermore, approximately 50% of cases develop metastasis within 5 years, urging the identification of biomarkers to assist prognostication and the development of more effective targeted therapeutics [4].

To meet this need, we have recently addressed the expression of the cancer-associated sialyl-Tn antigen (STn) on a small prospective series of unselected bladder cancer patients [5]. STn is an abnormal post-translational modification that results from a premature stop in cell-membrane proteins *O*-glycosylation by sialylation of the Tn antigen (Fig 1A). In bladder tumours, STn it was mainly present in advanced stage cases, while absent from most low-grade NMIBC [5]. Moreover, it was not expressed by the normal urothelium, denoting a cancer-specific nature [5]. Studies *in vitro* showed that STn expression endowed bladder cancer cells with high invasion capability [5] and an immunotolerogenic phenotype, potentially favoring disease dissemination [6]. Alterations in cell-surface protein glycosylation have be implicated in the activation of intracellular oncogenic signalling pathways [7], including the phosphoinositide-3 kinase (PI3K)/Akt signalling pathway [8] which is thought to play a critical role in bladder cancer development. These preliminary observations support the hypothesis that STn expression may play a key role in disease outcome, which warrants a deeper investigation. Several studies also suggest that Tn antigen, which is a precursor of STn, may be also implicated in oncogenic events [7]; however nothing is known about the expression of this glycan in bladder tumours.

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) pathways are interconnected signaling cascades essential for bladder cell growth and survival (Fig 1B). The PI3K/Akt/mTOR or mTOR pathway integrates a multiplicity of extracellular signals to regulate downstream signaling and protein synthesis, which ultimately leads to



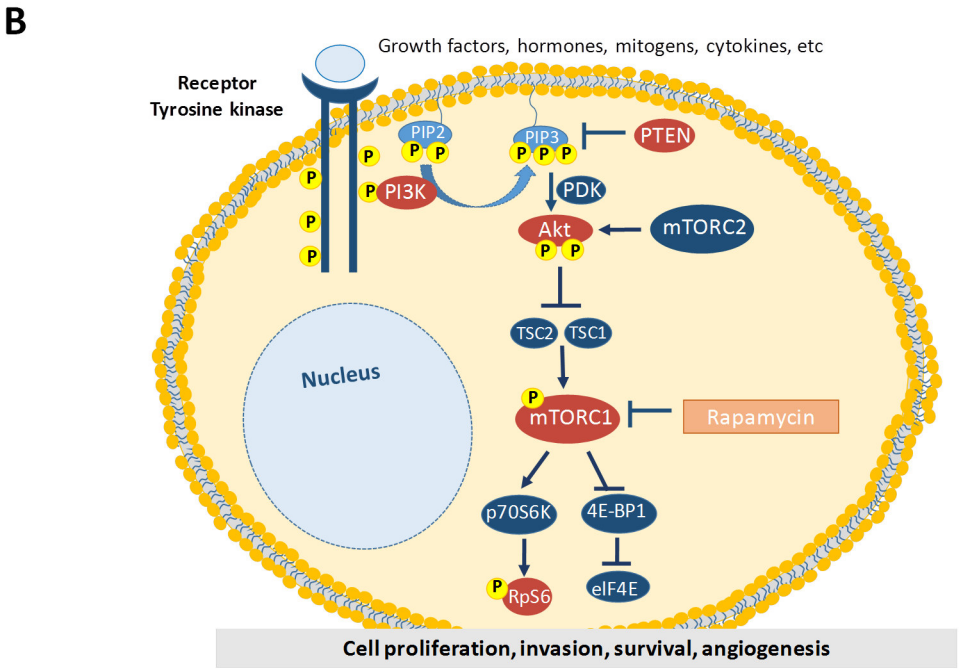


Fig 1. Schematic representation of membrane protein *O*-glycosylation and the PI3K/Akt/mTOR pathways. A) Representation of membrane protein *O*-glycosylation with emphasis on the STn expression by cancer cells. This is a highly regulated process of critical importance for protein stability and function. Briefly, newly synthesized proteins are *O*-glycosylated in the Golgi apparatus by the ppGalNAcTs-mediated addition of GalNAc moiety to Ser/Thr residues. This originates the Tn antigen (GalNAc-O-Ser/Thr-protein backbone), which is the simplest O-glycan. In normal cells these chains are extended through the sequential addition of other sugars first by CGALT-1 and then other enzymes. This culminates in highly complex, heterogeneous and elongated glycans often terminated by ABO or Lewis blood group related antigens (left drawing). In cancer cells the Tn antigen is immediately sialylated by ST6GalNAc.I, originating the STn antigen (Neu5Ac-GalNAc-O-Ser/Thr-protein backbone), thereby inhibiting further chain elongation (right drawing). The expression of STn at the cell surface influences cell-cell adhesion and cancer cell recognition, favouring motility, invasion and immune escape. B) Schematic representation of the PI3K/Akt/mTOR pathway, which is ubiquitously activated in bladder tumours. This is a highly conserved pathway regulated mainly by a wide variety of extracellular signals, including mitogenic growth factors, hormones, nutrients, cellular energy levels, and stress conditions. These signals activate tyrosine receptor kinases that recruit PI3K, which catalyses the conversion of membrane-bound PIP2 to PIP3. Then Akt and PDK-1 are activated through binding to PIP3. PTEN preferentially dephosphorylates PIP3, inhibiting signalling progression. Full Akt activation requires double phosphorylation by PDK-1 itself and PDK-2 (not shown). Akt phosphorylates mTOR directly or may also inactivate TSC1/TSC2 complex, inhibiting mTOR inactivation. mTORC1 triggers cell growth and proliferation by phosphorylating eukaryotic translation regulators, among these p70S6 kinase (p70S6K or S6K1) that, in turn, phosphorylates the

ribosomal protein S6 (pS6), and the eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1). For the protein mTOR to activate its signalling cascade, it must form the rapamycin-sensitive ternary complex mTORC1. Key PI3K/Akt/mTOR-pathway proteins pAkt, pmTOR and pS6 explored in this studied are highlighted by orange circles.

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a competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance [9]. The signaling cascade begins with PI3K activation in the cell membrane followed by serine/threonine kinase Akt cell membrane translocation and activation. The best studied downstream substrate of Akt is the serine/threonine kinase mTOR, whose downstream effector is S6 kinase-1 (S6K1). In particular, a subset of mTOR pathway alterations have been shown to occur in bladder cancer, such as mutations in *PIK3CA* gene, which culminates with increased mTOR signaling and bladder cancer cells resistance to apoptosis [10]. Moreover, the pharmacological or biochemical inhibition of the PI3K pathway drastically reduced the invasive capacity of bladder cancer cell lines. Furthermore, over half of primary human bladder tumours present high Akt phosphorylation and the aberrant activation of this pathway has been suggested to contribute to invasion [11]. Another event influencing mTOR pathway activation in bladder tumours involves the loss of tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) function [12]. PTEN normally suppresses activation of the PI3K/Akt/mTOR pathway antagonizing PI3K and preventing activation of Akt and PDK-1. PTEN also functions to regulate chemotaxis and cell motility, thereby promoting tumor invasion [13]. In summary, there are evidences that a comprehensive evaluation of PI3K/Akt/mTOR pathway associated proteins may hold significant potential for value for patient stratification. Moreover, many preclinical and clinical studies support that mTOR inhibitors, such as sirolimus (rapamycin) and their derivatives may improve cancer treatment [13,14].

Based on these observations we hypothesize that Tn and/or STn may act synergistically with the mTOR pathway to drive bladder cancer progression. As such, we have devoted to evaluating the expression of STn and proteins associated with the activation of the PI3K/Akt/mTOR pathway activation in bladder tumours at different stages. We anticipate that the combination of extracellular and intracellular oncogenic events may improve patient stratification and provide insights for novel therapeutics. Furthermore we have estimated the impact of sirolimus in chemically-induced urothelial tumours in mice, envisaging the creation of a rationale for more effective bladder cancer therapeutics.

Materials and Methods

Ethics Statement

This work involves experiences in tumour samples of patients diagnosed with bladder cancer in the Portuguese Insitute of Oncology of Porto. All procedures were performed after patient's written informed consent and approved by the Ethics Committee of Portuguese Institute of Oncology—Porto. All clinicopathological information was obtained from patients' clinical records.

It also involves animal experiments. All procedures involving animals were performed in accordance with the European Directive 2010/63/EU. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation (Direção Geral de Veterinária, Approval no. 520/000/000/2003). All mice used in the experiment were acclimatized for one week under routine laboratory conditions before starting the experiments. They were housed randomly in groups of 4–5 in plastic cages, with hard wood chips for bedding. The animals were maintained in a room with a controlled

temperature of 23±2°C, a 12-hour light/dark cycle and 55±5% humidity. The animals' drinking solutions were changed once a week or earlier if necessary, and the volume drunk was recorded. Weekly food intake was also noted. All mice were monitored throughout the experiment for signs of distress and loss of body weight. The animals were sacrificed with 0.4% sodium pentobarbital (1 ml/Kg, intraperitoneal).

Population

This study was performed in a retrospective series of 96 formalin-fixed paraffin-embedded bladder tumours obtained from archived paraffin blocks at the Portuguese Institute of Oncology—Porto (IPOP), Portugal. Bladder tumours were extracted from 82 men and 14 women, ranging in age from 38 to 92 years (median of 69.5 years), admitted and treated at the IPOP between 2005 and 2007. Forty seven of the examined tumours were histologically classified as NMBIC (Ta and T1) and 49 as invasive lesions (T2-T4). Sixteen were low grade and 80 were high grade tumours, according to the 2004 WHO grading criteria. Furthermore, carcinoma *in situ* (CIS) was found concomitantly in 20.8% of the patients. The average follow up time period was 45 months (1–134 months). Cystectomy was performed in 64 patients (66.7%) while the other 32 (33.3%) were submitted to transurethral resection. Lymphadenectomy was performed in approximately 47% of the patients and from those 37% presented metastasis. Fifty four (56.3%) tumours were primary and 42 (43.7%) were recurrent tumors. From the recurrent tumours, 38% had no prior treatment, 27% were treated with Mitomicin C, 11% with BCG and 19% were submitted to both treatments. Moreover 5% of these patients were treated with neoadjuvant chemotherapy prior to the cystectomy. Table 1 summarizes the clinicopathological information.

Cancer-specific survival (CSS) was defined as the period between the tumour removal by surgery and either patient death by cancer or the last follow-up information. All procedures were performed after patient's informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients' clinical records.

Immunohistochemistry

The expressions of STn antigen, its precursor Tn, and phosphorylated forms of Akt (pAkt), mTOR (pmTOR), S6 (pS6) and PTEN in bladder tumours were accessed by immunohistochemistry using the avidin/streptavidin peroxidase method, as described by Ferreira et al. [5]. Information on the primary antibodies and dilutions used in this study are summarized in Table 2. Immunoreactivity was revealed using diaminobenzidine (DAB, Thermo Scientific LabVision) as chromogen and sections were counterstained with Harris's hematoxylin. Negative controls were performed by replacing the primary antibody with 5% bovine serum albumin (BSA). Positive controls were known positive tissues for the antigens under study.

Immunohistochemistry scoring of human tumours

The immunostained sections were assessed double-blindly by light microscopy by two independent observers (CC and SP) and validated by an experienced pathologist (TA). Disaccording readings were re-analyzed using a double-headed microscope (Olympus BX46; Olympus Corporation), and consensus was reached. A semi-quantitative approach was established to score the immunohistochemical labeling based on the extent and intensity of the staining.

Given the absence of Tn and STn in the healthy urothelium [5], tumours were classified as positive for these antigens when membrane and/or cytoplasmic immunoreactivity were observed in more than 5% of the tumour, as described by Ferreira et al. [5,15]. pAkt, pmTOR, pS6 and PTEN expressions were scored according to the staining intensity (weak-1 point;

Table 1. Clinical-pathological data of the studied sample (*n* = 96).

Age, *years*		
	median [min—max]	69.5 [38–92]
Gender, *n* (%)		
	Male	82 (85.4%)
	Female	14 (14.6%)
Stage, *n* (%)		
	Ta	27 (28.1%)
	T1	20 (20.8%)
	T2	9 (9.4%)
	T3	20 (20.8%)
	T4	20 (20.8%)
Grade, n (%)		
	Low	16 (16.7%)
	High	80 (83.3%)
Recurrence status, n(%)		
	Primary	54 (56.3%)
	Recurrent	42 (43.7%)
Associated Cis, n(%)		
	No	76 (79.2%)
	Yes	20 (20.8%)
Metastasis, n(%)		
	No	19 (63.3%)
	Yes	11 (36.7%)
Follow-up, *n (%)*		
	Alive, lost or death from other causes	67 (69.8%)
	Death from cancer	29 (30.2%)

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moderate-2 points; strong-3 points) multiplied by the percentage of positive cells (0–5%-0 points; >5–25%-1 point; >25–50%-2 points; >50–75%-3 points; >75–100%-4 points). Based on the classification proposed by Nishikawa et al. [16], tumours with a score <6 were considered negative, whereas those with a score≥6 were classified as positive (overexpression). pAkt was evaluated based on nuclear immunoreactivity, pmTOR and pS6 based on cytoplasmic expression and PTEN on both cytoplasmic and nuclear staining, as suggested by other publications [17,18].

Animal experiments with sirolimus and immunohistochemistry scoring

Histological sections of Imprinting Control Region (ICR) mice bearing N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder lesions, resulting from our previous work on the impact of sirolimus on bladder cancer [19], were elected for this study. Briefly, four-week-old male ICR mice (25g; Harlan, Barcelona, Spain) were randomly distributed into four groups, as described in detail in a previous publication [18]. Group 1 (n = 6) included mice exposed to 0.05% BBN for 12 weeks followed by tap water for 8 weeks (total of 20 weeks). Group 2 (n = 7) included mice treated with 0.05% BBN solution for twelve weeks, maintained with normal tap water for another week, administrated intraperitoneally with mTOR-inhibitor sirolimus (1.5 mg/kg; Wyeth) for five days a week for six consecutively weeks, i.e. until the 19th week, followed by another week of tap water (total of 20 weeks). Group 3 (n = 6) included mice exposed to 0.05% BBN for 12 weeks followed by tap water for 11 weeks (total of 23 weeks). Group 4

Table 2. Antibodies used in the immunohistochemical analysis.

Antibody	Vendor	Clone	Dilution
Tn	Non-commercial Hybridoma*	IE3	1:5
STn	Non-commercial Hybridoma*	TKH2	1:20
Ki-67	Dako	MIB-1	1:100
p53	Dako	DO-7	1:100
Phos-AKT	Cell Signaling	Ser473 (736E11)	1:50
Phos-mTOR	Cell Signaling	Ser2448(49F9)	1:100
Phos-S6	Cell Signaling	Ser240/244 polyclonal	1:75
PTEN	Cell Signaling	D4.3 XP	1:50

*Kindly provided by Prof. Celso Reis (IPATIMUP, UP, Portugal)

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(n = 7) included mice treated with 0.05% BBN and sirolimus, as described for Group 2, but with an exposure to tap water afterwards of 3 weeks (total of 23 weeks). Group 3 and 4 were created to estimate the possibility of late relapse and/or molecular alterations resulting from prolonged survival. All procedures were performed in accordance with the European Directive 2010/63/EU. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). The histological changes induced by these experiments included both preneoplastic and neoplastic lesions with invasive potential and invasive tumours, as described in detail by Oliveira et al. [18]. Herein, lesions of high invasive potential and muscle invasive tumours were screened for STn and pS6 by immunohistochemistry, as described in detail for human tumours, since the antibodies used are reactive against both human and mice. Both the intensity and the extension of immunostaining were taken into consideration to score the expression of the antigens, as described in the previous section. The bladder lesions and immunostaining were assessed double-blindly by two independent observers (CC and SP) and validated by an experienced veterinary pathologist (RMGC).

Statistical analysis

Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0). Chi-square analysis was used to compare categorical variables. Kaplan-Meier survival curves were used to evaluate correlation between STn expression and cancer-specific survival (CSS) and were compared using log-rank test. Furthermore, multivariate Cox regression analysis was performed to assess the individual effect of the evaluated markers on patient's survival and adjust to potential confounders (variables that could affect CSS of NMIBC and MIBC patients). The correlation between PI3K/Akt/mTOR pathway molecules was performed using Spearman rho test.

Results

Altered protein glycosylation, translated by the expression of the STn antigen and its precursor Tn, PI3K/Akt/mTOR pathway molecules (pAkt, pmTOR, pS6), and PTEN inactivation, are salient features of bladder tumours. Herein we have devoted to a comprehensive analysis of these molecular alterations in a series of bladder cancer patients at different stages of the disease, envisaging biomarkers of poor cancer-specific survival.

Our dataset was composed by 47 NMIBC and 49 MIBC patients, as showed in Table 1. According to Fig 2, NMIBC presented a higher cancer-specific survival (CSS; mean CSS: 119 months) than MIBC patients (mean CSS: 43 months; log rank, $p<0.001$). These results

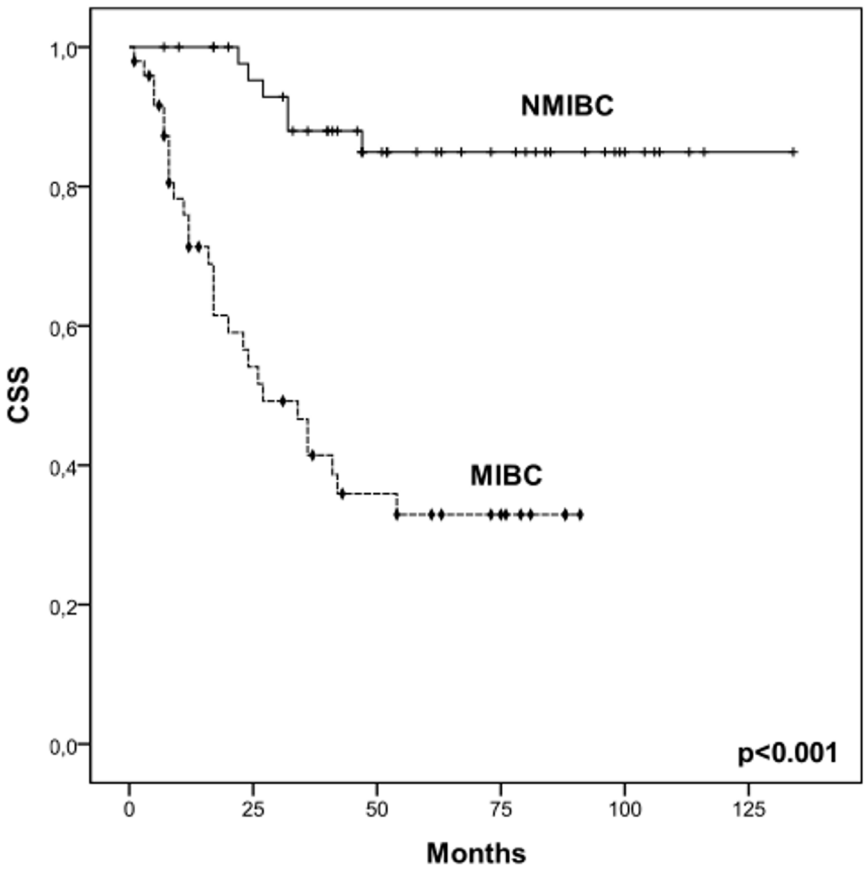

Fig 2. Association between disease groups and cancer-specific survival (CSS) in the studied patients. Kaplan-Meier analysis showing the CSS of NMIBC (Ta and T1) and s of MIBC (T2,T3 and T4). Comparison performed by log-rank test (p<0.001); + censored NMIBC patients; ♦ censored MIBC patients.

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demonstrated that our series reflected the natural course of disease and highlighted the significantly lower CSS of MIBC compared to NMIBC cases. Therefore, particular interest was set in the identification of biomarkers for late stage disease based on the comparison between NMIBC and MIBC.

Tn and STn antigen expressions in bladder cancer

The Tn antigen was observed in approximately 10% of NMBC and MIBC (Table 2) and its expression was residual, did not exceeding 5% of the tumour area and without any defined pattern. On the other hand, the STn antigen was detected in approximately 60% of the studied bladder tumours, which is in accordance with our previous findings [5]. The antigen was predominately expressed at the cell membrane, although cytoplasmic staining could also be observed. The STn antigen presented a focal expression that did not exceed 30% of the tumour area for the majority of the positive cases, irrespectively of their histological origin. STn was mainly expressed by dedifferentiated cells in tumours showing *lamina propria* (T1; 60%) and *muscularis propria* (≥ T2; approximately 60–90%) invasion; conversely the percentage of positive Ta was lower than 30% ($p<0.001$; Fig 3A). Although without statistical significance, in Ta tumours STn positive cells were mainly present in superficial tumour layers away from the vessels. Conversely, STn positive cells in T1 tumours (Fig 3B) were observed accompanying and/

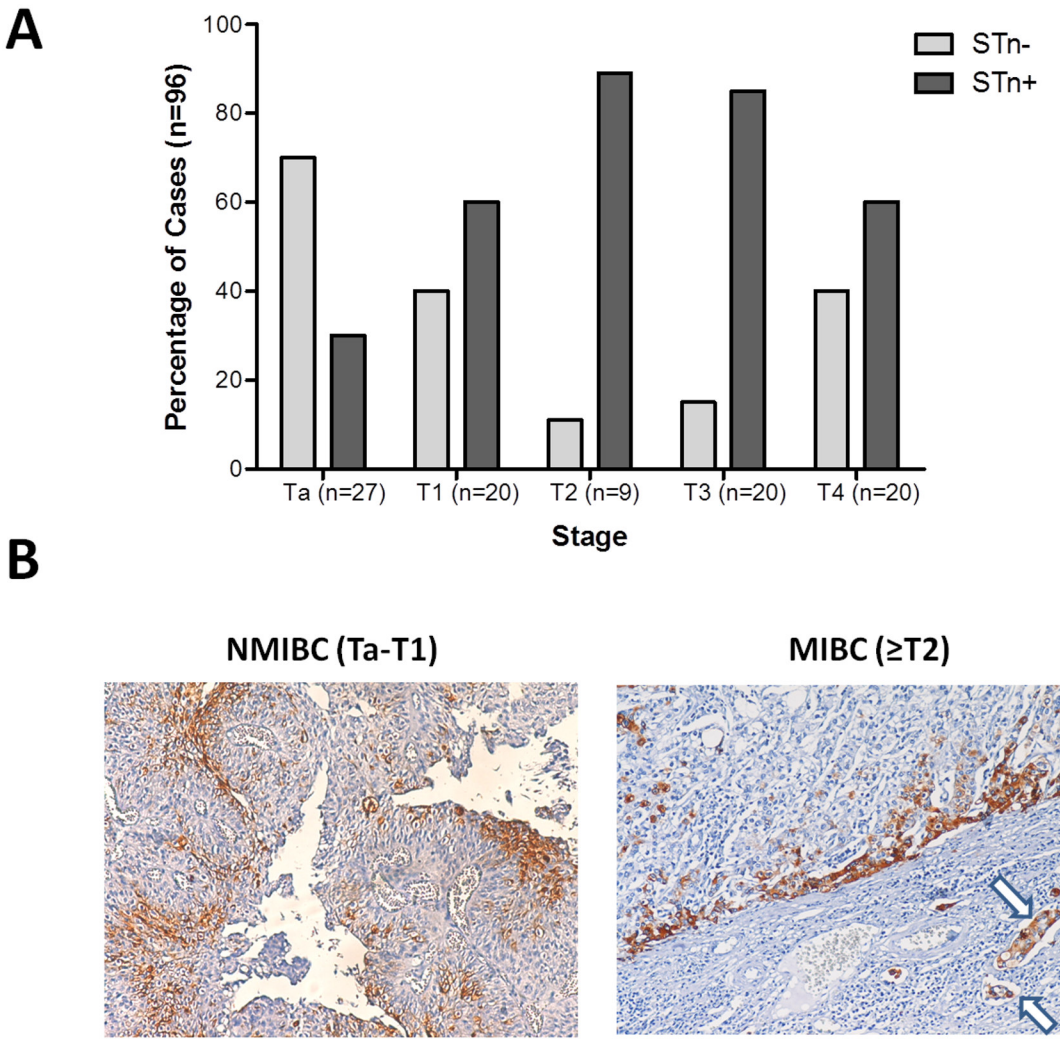

Fig 3. STn expression in different bladder tumors stages. (A) Distribution of STn negative and positive tumors along the different stages of bladder cancer; (B) Representative images of STn staining in NMIBC and MIBC. Left—NMIBC showing a predominance of STn positive cells in the superficial layers, away from the fibrovascular support; note vessels without positive cells. Right—MIBC showing the invasion front with STn positively stained cells; note positive STn urothelial cells in the vessels (arrow), suggesting possible involvement in metastasis.

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or invading the basal layer (Fig 3B), while in MIBC these cells were mostly found in the invasion fronts (Fig 3B) and invading and/or inside the vessels, which suggests a role in invasion and disease dissemination. Reinforcing these observations, the presence of STn antigen was statistically higher in MIBC when compared to NMIBC ($p = 0.001$, Table 3).

PI3K/Akt/mTOR pathway in bladder cancer

The evaluation of the PI3K/Akt/mTOR/S6 pathway was done using antibodies for active phosphorylated forms of Akt (pAkt), mTOR (pmTOR), and S6 (pS6). PTEN, that negatively regulates Akt signalling, was also evaluated.

pAkt was detected both in the cytoplasm and nucleus. In NMIBC cases several areas with different intensity of expression were observed (Fig 4A), denoting a heterogeneous pattern that was not evident in MIBC (Fig 4B). Furthermore, stromal cells of MIBC positive cases showed enhanced staining intensity mainly in the areas close to the tumour. pmTOR immunoreactivity

Table 3. Association between the evaluated markers and the stage of disease.

	Bladder Cancer		
	NMIBC n (%)	MIBC n (%)	P
Tn			
Negative	41 (87.2)	45 (91.8)	
Positive	6 (12.8)	4 (8.2)	0.461
STn			
Negative	27 (57.4)	12 (24.5)	
Positive	20 (42.6)	37 (75.5)	0.001
pAKT			
Negative	13 (28.9)	19 (38.8)	
Positive	32 (71.1)	30 (61.2)	0.312
pmTor			
Negative	30 (63.8)	33 (67.3)	
Positive	17 (36.2)	16 (32.7)	0.717
pS6			
Negative	22 (47.8)	28 (57.1)	
Positive	24 (52.2)	21 (42.9)	0.183
PTEN			
Negative	18 (38.3)	37 (82.2)	
Positive	29 (61.7)	8 (17.8)	<0.001

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was cytoplasmic and, in occasional cases, nuclear. In urothelium with apparent normal histology pmTOR expression was restricted to superficial cell layers. In NMIBC pmTOR expression was evenly distributed across the several layers of urothelial cells, although there was a more intense staining in the superficial layers (Fig 4C). Moreover, several areas with variable staining intensity were observed, denoting a heterogeneous expression. In MIBC positives cases, pmTOR expression was focal and heterogeneous (Fig 4D). pS6 immunoreactivity was predominantly cytoplasmic. In NMIBC pS6 expression was noted in all the superficial layers, both in umbrella and differentiated cells (Fig 4E). The immunoreactivity of pS6 varied across the tumour cells. In MIBC pS6 presented a diffuse expression throughout the tumour, being more present in basal and mitotic cells (Fig 4F). Several positive cases presented increased pS6 staining intensity in the invasion front as well as pS6 expression in tumour infiltrating lymphocytes and endothelial cells.

Taking into account the extension of staining and its intensity, 62/94 (66%), 33/96 (34%) and 45/95 (47%) of the bladder tumours were considered positive for pAkt, pmTOR and pS6, respectively. A Spearman rho test showed that pAkt, pmTOR, pS6 expressions were significantly correlated ($P<0.05$) irrespectively of the tumour stage, thus in accordance with a fully active pathway. Furthermore, despite histological differences, these markers presented an equal distribution among the NMIBC and MIBC and could not be associated with muscle invasion (Table 3).

On the other hand, 37/92 (40%) of the tumours were considered positive for PTEN. PTEN was expressed in the cytoplasm and nucleus of the same cells, however with lower extension of expression in MIBC (33%, Fig 4G) compared to NMIBC (83%; Fig 4H). Moreover, the PTEN-negative phenotype was significantly associated with muscle invasion (Ta and T1; $p<0.001$, Table 3), which may contribute to maintain an active PI3K/Akt/mTOR/S6 pathway in these cases.

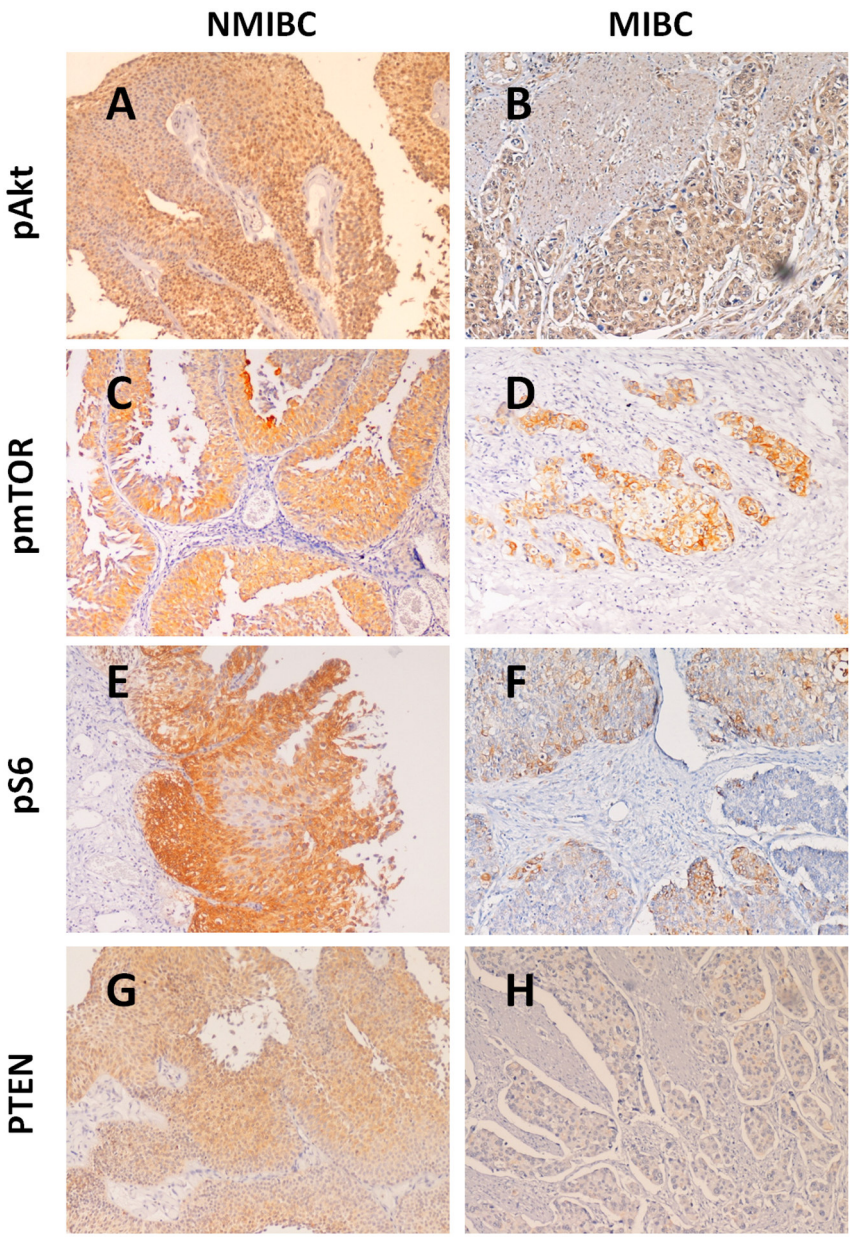

Fig 4. Expressions of pAkt, pmTOR, pS6 and PTEN in NMIBC and MIBC (40x magnification). A and B) pAKT nuclear and cytoplasmatic expression in NMIBC (A) and MIBC (B). In NMIBC cases pAkt presented a heterogeneous pattern with areas of different intensity of expression. In MIBC, stromal cells mainly in the areas close to the tumour showed higher expression. C and D) pmTOR cytoplasmic expression in NMIBC (C) and MIBC (D). In NMIBC pmTOR was expressed across several layers, although there was a more intense staining in the superficial ones. In MIBC positive cases pmTOR expression was focal. E and F) pS6 cytoplasmatic expression in NMIBC (E) and MIBC (F). In NMIBC pS6 expression was observed in all the superficial layers both in umbrella and differentiated cells. In MIBC the immunoreactivity was diffuse, however more present in basal and mitotic cells. pS6 expression was higher in the invasion front and in tumour infiltrating lymphocytes and endothelial cells. G and H) PTEN cytoplasmic and nuclear expressions in NMIBC (G) and MIBC (H). PTEN expression was higher in NMIBC compared to MIBC.

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Tn, STn, PI3K/Akt/mTOR pathway and Cancer-specific Survival

A Kaplan-Meier analysis was used to evaluate associations between the addressed biomarkers and the cancer-specific survival of patients. We observed that patients bearing STn expressing tumours had a lower CSS, irrespectively of their stage ($p = 0.024$; Fig 5A). This was also observed when evaluating NMIBC alone ($p = 0.020$; Fig 5B). More importantly, among NMIBC, STn expressing T1 tumours presented lower CSS than negative tumours ($p < 0.05$). Moreover, multivariate Cox regression analysis adjusted to potential confounders, namely age, gender, stage, grade, recurrence status, presence of concomitant CIS was performed. We found that STn is an independent prognostic marker of worst CSS (HR = 11.836; 95%CI: [1.063–131.7]; $p = 0.044$). Contrasting with STn, positive Tn, pAkt, pmTOR and pS6 tumours showed no differences in CSS compared to negative lesions, irrespectively of their stage. We have also observed that patients harbouring PTEN-negative tumours had lower CSS ($p = 0.015$, Fig 6). More studies are necessary to determine if the lack of suppressive effect of PTEN over PI3K/Akt/mTOR may account for these findings.

Based on these observations and aiming to improve the prognostic value of STn in the context of late stage disease (MIBC), we have comprehensively integrated the information from STn and PI3K/Akt/mTOR pathway biomarkers. According to Fig 7, the introduction of PI3K/Akt/mTOR pathway molecules allowed discriminating STn positive MIBC tumours with worst CSS ($p = 0.027$). Furthermore, multivariate Cox regression analysis (adjusted to age, stage, recurrence status, presence of concomitant CIS and metastasis) revealed that the presence of PI3K/Akt/mTOR pathway molecules in STn+ MIBC is independently associated with approximately 6-fold risk of death by cancer (HR = 5.662; 95%CI: [1.093–29.323]; $p = 0.039$). These observations suggest, for the first time, that the combination of STn and mTOR pathway biomarkers may hold potential to improve the stratification of advanced stage bladder tumours; however corroboration in larger series is mandatory.

Inhibition of the PI3K/Akt/mTOR pathway in animal models

BBN-induced mice bladder tumours mimicking the histology and molecular nature of human cancers [20,21], were screened for STn and pS6, the downstream effector of mTOR pathway. We observed no STn expression in the healthy mice urothelium, in accordance with previous observation for the healthy human bladder [5]. In mice healthy urothelium pS6 expression was below 20%, thus underexpressed when compared with BBN-exposed mice (Fig 8). In the control groups (Group 1 and 3, Fig 8A), the exposure to BBN led to the development of invasive tumours in 70–90% of the studied mice. Concomitantly, 83–100% of the invasive lesions overexpressed the STn antigen and all significantly overexpressed pS6 (Fig 8B). This demonstrated that BBN-induced lesions were able to recapitulate the association between altered glycosylation and an activated PI3K/Akt/mTOR pathway previously observed in advanced stage human tumours. The STn antigen was mainly found in cells adjacent to the basal layer and in those invading the stroma, as previously observed in human tumours (Fig 8B and 8C). Conversely, pS6 presented a more diffuse expression, again in accordance with the pattern observed in human lesions (Fig 8B and 8C). A comparison between groups 1 and 3 further highlighted that extended lifespan did not alter the number of invasive lesions, but significantly increased STn and pS6 overall expressions in each tumour ($p<0.05$; Fig 8B), highlighting the more aggressive nature of Group 3 lesions.

In the sirolimus-treated groups (Groups 2 and 4; Fig 8A) a smaller number of mice developed invasive tumors (20–40%) when compared to the controls (Groups 1 and 3). Moreover, only 43% of the mice treated with sirolimus overexpressed the STn antigen, irrespectively of the experience periods. Still, the extension of STn expression was significantly decreased in

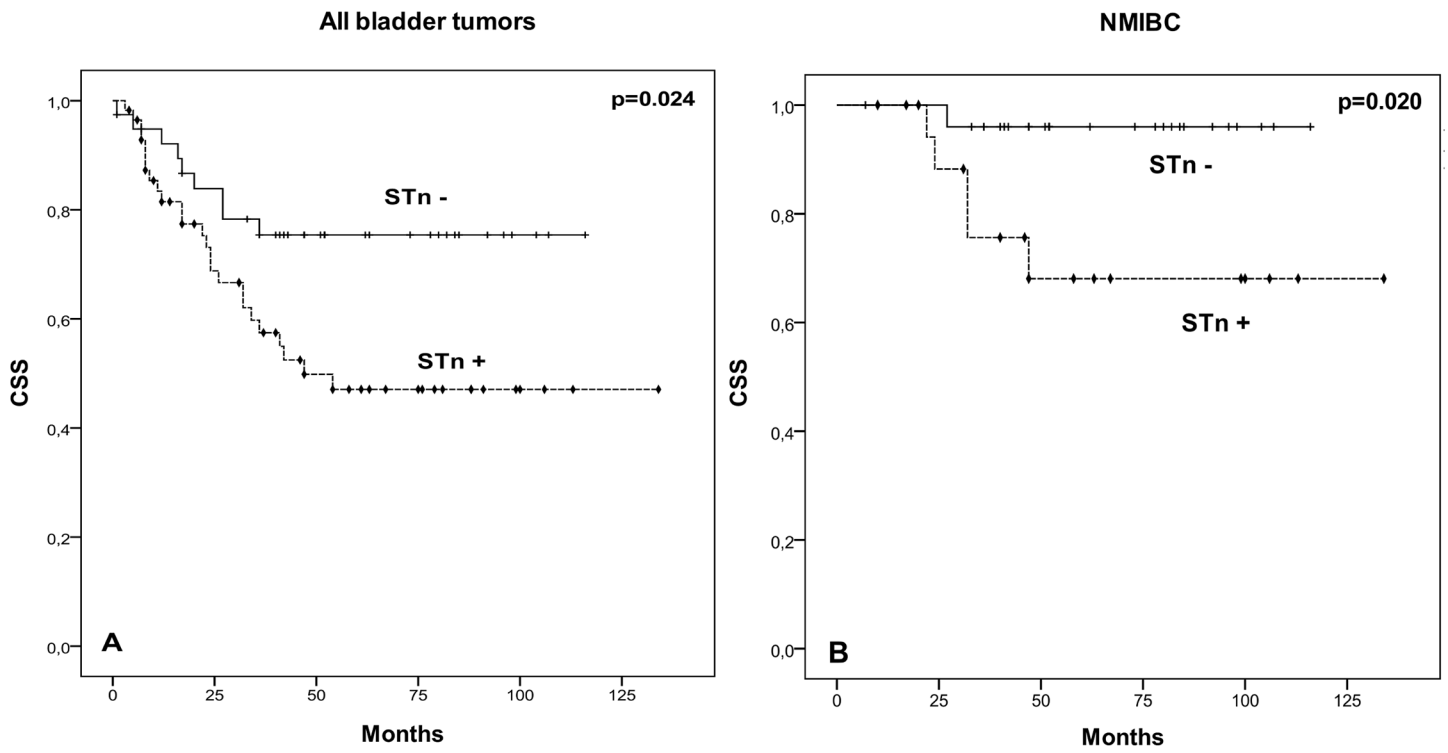

Fig 5. Effect of STn expression in cancer-specific survival (CSS). Kaplan–Meier analysis showing the association between STn and CSS in: (A) all studied bladder cancer patients; (B) NMIBC patients. Comparison performed by log-rank test (A: p = 0.024; B: p = 0.020); + censored STn negative tumours; ♦ censored STn positive tumours.

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STn-positive tumours when compared to the control groups (Fig 8B and 8C). Following the same tendency, the pS6 protein was only overexpressed in 29% of the cases in Group 2 and the extension of expression was also significantly decreased (Fig 8B and 8C). Contrastingly, the expression of pS6 in Group 4 was higher than in Group 2, again translating the higher aggressive nature of tumours obtained after longer lifespan. Despite these observations, sirolimus treatment promoted a significant reduction in the percentage of positive pS6 cells in Group 4 mice when compared to Group 3 ($p<0.05$; Fig 8B and 8C). Altogether, sirolimus administration effectively reduced tumour burden and promoted a significant reduction in the expression of STn and pS6 markers.

Discussion

Due to their high molecular heterogeneity, advanced stage bladder tumours present a significant prognostication and treatment hurdle. In this context, much controversy exists regarding the potential of conventional cancer biomarkers, urging the identification of novel molecules capable of aiding disease personalization. Furthermore, advanced stage bladder cancer remains an orphan disease in terms of therapeutics, as the only available options continue to be surgery and conventional chemotherapy [22]. The introduction of targeted therapeutics is therefore warranted.

In a previous explorative study we have observed that altered protein glycosylation translated by STn overexpression was a salient feature of a subset of advanced stage tumours [5]. Herein we have started by investigating the expression of STn precursor, the Tn antigen, in bladder tumours. We observed that this antigen presented a very low expression in bladder

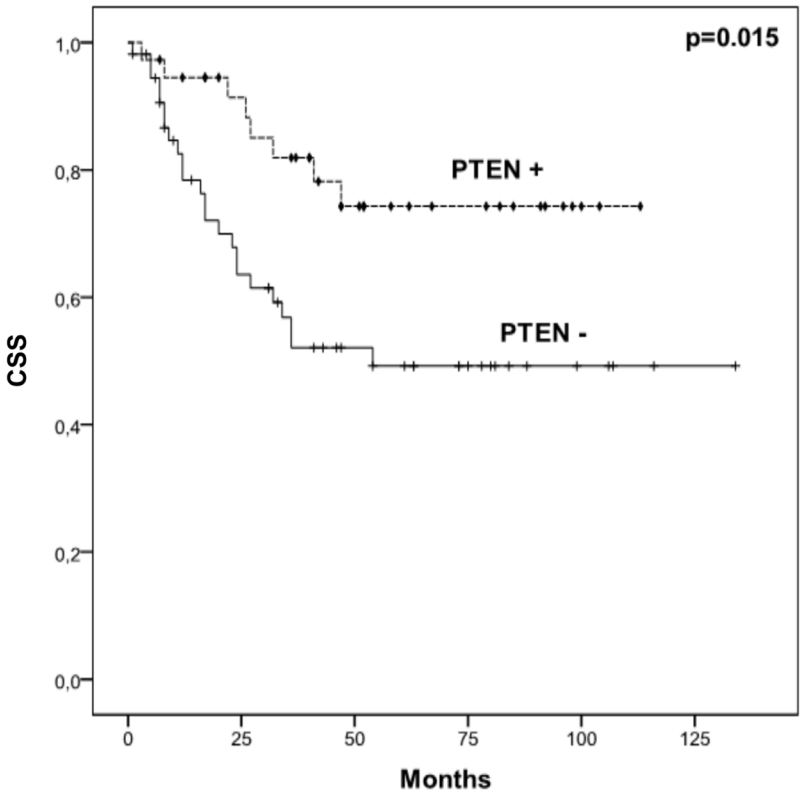

Fig 6. Effect of PTEN expression and cancer-specific survival (CSS) in the studied patients. Kaplan-Meier analysis showing the effect in CSS of PTEN expression in all studied bladder cancer patients. Comparison performed by log-rank test (p = 0.013); + censored PTEN negative tumours; ♦ censored PTEN positive tumours.

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tumours and was not associated with any particular stage of the disease. These findings suggest that the Tn antigen is rapidly sialylated or capped with more extended glycans in bladder tumours. Moreover, we have confirmed that STn expression is more associated with muscle invasive than non-muscle invasive disease in a larger patient set, suggesting that sialylation plays a key role in stopping protein glycosylation in advanced stage bladder tumours. Furthermore, we have provided new insights regarding its correlation with decreased survival, as previously observed for digestive track tumours [23–25]. Accordingly, we and other authors have shown that STn expression is responsible by the modulation of cell surface glycoprotein functions in ways that favour malignant phenotypes in gastric [26], breast [27] and bladder [5] cancers. Namely, STn expression altered the adhesive properties of cancer cells, possibly by impairing integrin function [26,27]. Furthermore, it enhanced cell motility, invasion [26,27] and epithelial-to-mesenchymal transition, a key event leading to metastasis [28]. We have also demonstrated that STn expression protects bladder cancer cells from adverse host immune responses [6]. Namely, it impaired dendritic cell maturation inducing a tolerogenic phenotype and limiting their capacity to trigger protective anti-tumour T-cell responses [6]. In resume, a significant amount of data supports a key role of STn in disease progression and dissemination, making of STn antigen, and in particular STn-glycoproteins, potential anti-cancer targets. Nevertheless, there is scarce information about the molecular nature of this subset of STn-expressing aggressive tumours and consequently about the best therapeutic options.

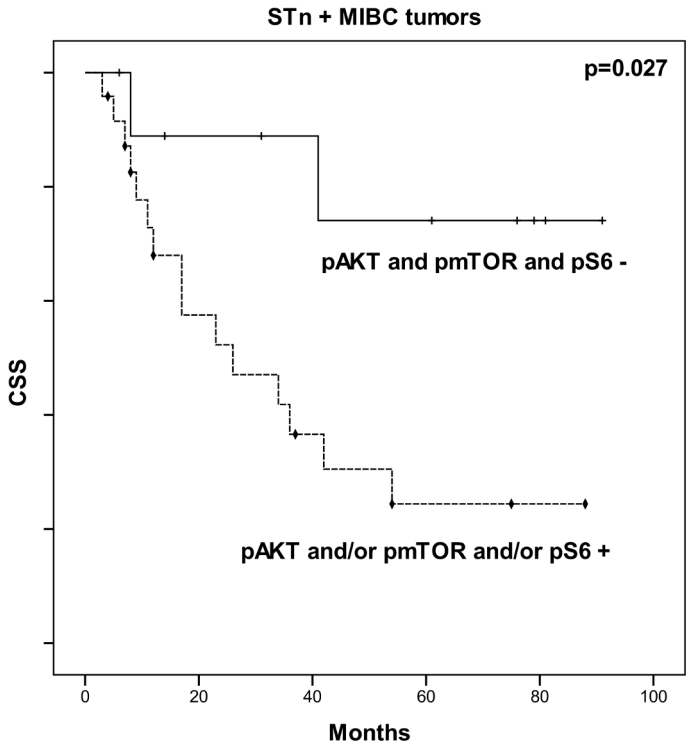

Fig 7. Effect of PI3K/Akt/mTOR pathway activation in cancer-specific survival (CSS) of patients with STn positive MIBC. Kaplan–Meier analysis showing the association between pAKT, pmTOR and pS6 expressions in the CSS of STn positive tumors MIBC: Comparison performed by log-rank test (p = 0.027); + censored pAKT and pmTOR and pS6 negative tumours; ♦ censored pAKT and/or pmTOR and/or pS6 positive tumours.

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Foreseeing a more accurate patient stratification we have also addressed the expression of PI3K/Akt/mTOR pathway markers in bladder tumours. In our series the activation of mTOR pathway proteins did not discriminate the stage of disease. Moreover it did not allow, by itself, the identification of patients facing worst prognosis, which is in accordance with recent publications [29,30]. However, we found that PTEN expression, which exerts a suppressive effect over the PI3K/Akt/mTOR pathway, was decreased in advanced stage tumours, in accordance with previous observations [31–34]. Furthermore, PTEN-negative MIBC presented worst cancer-specific survival in comparison to PTEN-positive lesions. More studies are needed to determine if the lack of suppressive effect over the PI3K/Akt/mTOR may account for poorer outcome. Interestingly, we have also observed that the overexpression of PI3K/Akt/mTOR pathway biomarkers decisively associated with worst CSS in STn positive advanced stage tumours, which currently lack effective therapeutics. These findings lead us to hypothesize that this subset of more aggressive bladder tumours may benefit from multi-targeted approaches combining mTOR-inhibitors and guided therapeutics against STn-expressing cells. However these are preliminary insights from a relatively low number of patients. More studies involving a large population are warranted to confirm these observations. It will also be important to evaluate other outcomes of aggressiveness, namely response to conventional therapeutics and metastasis development.

Our study also reinforced that bladder tumours present extensive activation of the PI3K/Akt/mTOR pathway irrespectively of their histological nature, as described in previous publications [32,35]. Such findings contribute to support the idea that most bladder tumours may be good candidates for mTOR-inhibitors therapeutics. Accordingly, mTOR-inhibitors have been

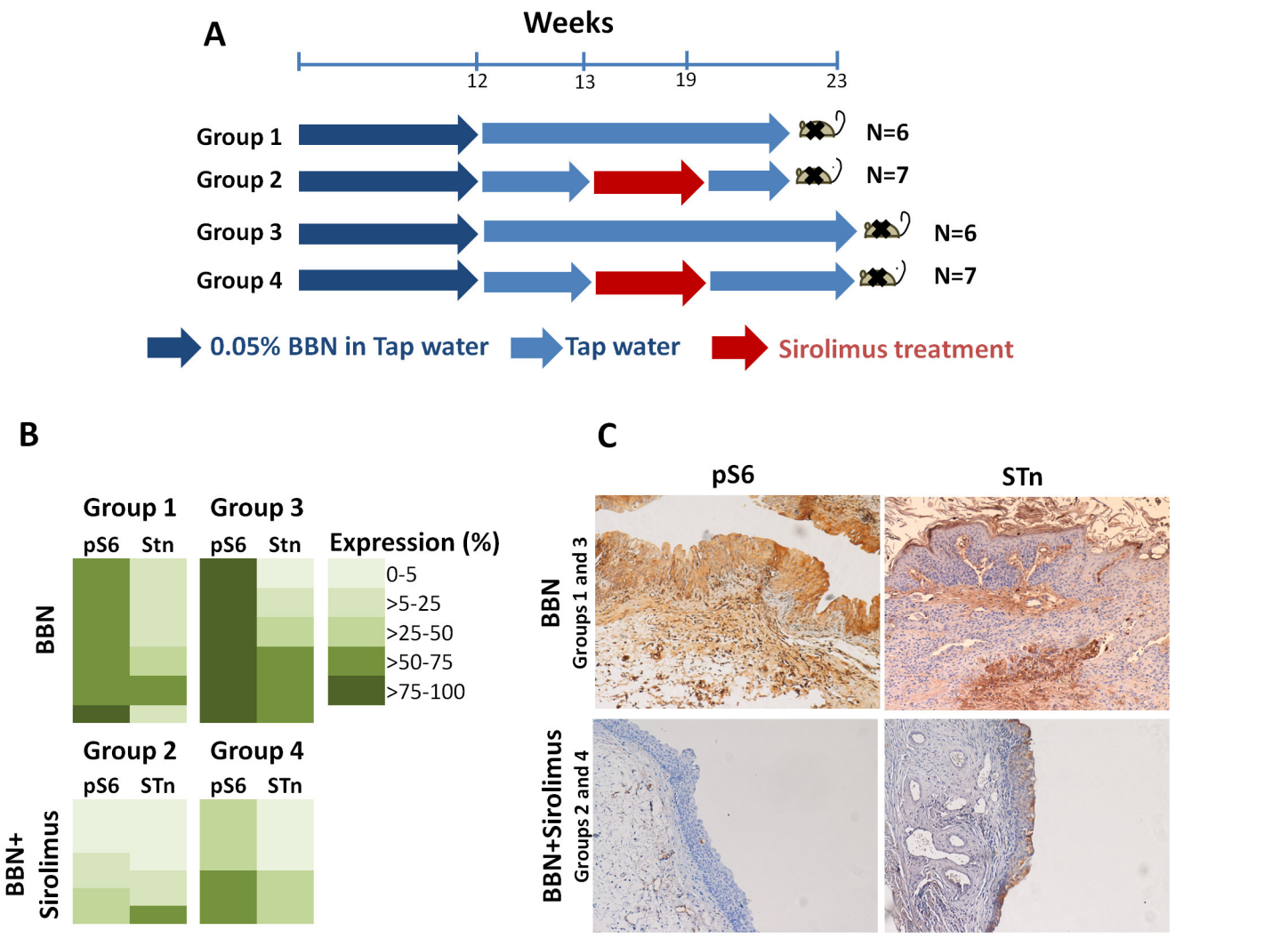

Fig 8. STn and pS6 expressions in bladder tumours from BBN-exposed male ICR mice with or without the administration of mTOR-inhibitor sirolimus (rapamycin). A) Experimental design to determine the sirolimus effect on STn and pS6 expressions in a model of urothelial carcinogenesis (male ICR mice). B) Expression of STn and pS6 in BBN-derived urothelial tumours in the presence and absence of sirolimus. BBN-induced bladder tumours (Groups 1 and 3) overexpressed STn and pS6, which was more pronounced in Group 3, after longer lifespan. Exposure to sirolimus decreased the number of invasive lesions in groups 2 and 4 (data not shown) and, concomitantly, decreased the expressions of STn and pS6. C) Histological sections showing the expressions of STn and pS6 in BBN-induced urothelial tumours before and after treatment.

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extensively explored in pre-clinical settings and two phase I/II clinical trials for bladder cancer are ongoing [36]. In particular our group has demonstrated that the combination of everolimus with cisplatin or gemcitabine decreased the proliferation of bladder cancer cell lines in comparison to the chemotherapy agent alone [14,37]. More recently we conducted studies in mice bearing chemically-induced tumours mimicking the histological and molecular nature of human tumours [20]. We concluded that administration of mTOR-pathway inhibitor sirolimus (rapamycin) effectively reduced the frequency of invasive lesions. Using the same animal model, we have now confirmed the anti-cancer activity of sirolimus in the context of aggressive bladder disease. Namely, we observed a significant reduction in tumour burden accompanied by a loss of pS6 expression, thus in accordance with the expected mechanism of action of the drug. Moreover, we are describing for the first time that chemically-induced bladder tumours expressed the

STn antigen, thereby mimicking the glycosylation pattern of human cancers. These observations are of particular importance due the lack of accurate models to access the biological role of this antigen. In fact most established cancer cell lines express residual amounts of this antigen, denoting a dependence on the tumours microenvironment. We believe that BBN-induced tumours may now constitute key models to develop successful therapeutics against STn positive bladder lesions. Moreover importantly, we have concluded that the administration of sirolimus contributed to reduce the number of STn positive cells. These observations reinforce a possible association between STn and an active PI3K/Akt/mTOR pathway in invasive tumours, as suggested upon the evaluation of human cancers. It also points out that sirolimus may constitute a valuable approach to manage STn and PI3K/Akt/mTOR-positive, which face worst OS. Still, these preliminary evidences and more in depth studies are needed before progressing to clinical phases. Namely, it will be important to support these findings in other models such as patient-derived xenografts and compare the effect of sirolimus with conventional chemotherapeutics for bladder cancer (cisplatin/gemcitabine-based regimens).

In resume, we have demonstrated that the STn antigen is a biomarker of poor prognosis, particularly in MIBC. We also suggest the existence of potentially more aggressive subgroup of STn positive MIBC characterized by an active mTOR-pathway. Such observations also provide the first link between these two apparently unrelated events in bladder cancer (altered glycosylation and the PI3K/Akt/mTOR-pathway activation). Using animal models we have also concluded that the administration of mTOR-pathway inhibitor sirolimus offers potential against these highly malignant tumours. More validation studies are now warranted to set the pace for clinical trials. Taking into consideration its cell-surface nature and key role played by STn malignancy, specific antibody-based therapeutics can also be envisaged [22,38]. The combination of these approaches may provide novel ways to improve MIBC management, which remains an orphan disease in terms of innovative treatments [22].

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Author Contributions

Conceived and designed the experiments: CC SP LL PO JAF LLS. Performed the experiments: CC SP AP EF AT DN MN CG RMGC PO. Analyzed the data: CC SP LL RMGC RC TA PO JAF LLS. Contributed reagents/materials/analysis tools: RC PO JAF LLS. Wrote the paper: LL JAF LLS PO.

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4. FINAL DISCUSSION

Bladder cancer can be classified into two groups: non muscle invasive (NMIBC - stages Ta, T1 and TIS) and muscle-invasive or metastatic bladder cancer (MIMBC; T2–T4). NMIBCs tend to recur but rarely progress to invasive cancer. On the other hand, MIMBCs have a high morbidity and mortality, in spite of the treatment. Clearly, objective biomarkers are needed in clinics to complement conventional clinicopathological markers to predict prognosis, treatment response and survival in both NMIBC and MIMBC patients.

STn is known to influence cell recognition by the immune system [132], affect processes as cell cycle, apoptosis, and actin cytoskeleton dynamics, decrease cell-cell aggregation and increase extra-cellular adhesion, migration, invasion [39,133,134,135] and metastization [89]. Overexpression of STn antigen has been detected in several human carcinomas and preneoplastic lesions [77], whereas low or no expression was observed in the respective normal tissues [84].
Despite the cancer-associated nature of the STn antigen, few studies have been presented for bladder cancer.

Within the line of this thesis, the first study (Paper I) aimed to understand the mechanisms underlying the presence of STn in bladder tumours. In this study, with 69 patients (50 NMIBC and 19 MIBC) STn was not expressed in the healthy urothelium (6 samples); however, 46% of the bladder tumours presented cells with STn membrane and/or cytoplasmic staining, demonstrating the tumour-specific nature of this antigen. The results showed that STn expression was lower in low-grade (LG) NMIBC (21% STn+ tumours) compared to high-grade lesions (HG; 67% STn+ tumours), which include papillary tumours (76% STn+ tumours), CIS (20% of STn+ tumours), and MIBC (74% STn+ tumours). Altogether, these results highlight an association between the STn antigen and high grade NMIBC ($p<0.002$) as well as with muscle invasive tumours ($p<0.03$).
Contrastingly, Langkilde (1992) reported no association between tumour progression to invasion and the expression of sialyl-Tn-antigen, which probably lead them to abandon this marker in bladder cancer [126]. However, *in vitro* studies have demonstrated that STn expression enhanced the invasion capability of bladder cancer cells [130]. Despite the controversy about the prognostic value of STn in bladder cancer, this marker was again target of research and, according

to the obtained results; it seems to be of promising biological value (Paper I). As so, STn may also be monitored noninvasively in urine or serum using the CA72-4 test as is the case of other human carcinomas [28]. This could probably allow decreasing the number of cystectomies in post-surgery follow-up of patients with high-grade tumours, preventing major morbidities.

It was known, from the previous study that STn was mainly expressed by HG papillary NMIBC, known for their elevated risk of recurrence and progression to muscle invasive disease and in MIBC that encompass an elevated risk of metastization and present decreased overall survival [22]. Further, that STn expression was also associated with elevated Ki-67, a proliferation-related molecule and a surrogate biomarker of increased risk to recurrence and progression in bladder tumours [136,137]. BCG is currently used for CIS and recurrent superficial disease of the bladder [15]. The response to intravesical treatment with BCG or chemotherapy is an important prognostic factor for subsequent progression and death caused by bladder cancer. Approximately 10-20% of complete responders eventually progress to muscle-invasive disease, compared with 66% of non-responders [22]. There are evidences that STn expression may modulate the cell–BCG interaction [39,134,138] as well as immune responses [94,99,139]

With the second paper (Paper II), it was hypothesize that STn might modulate BCG attachment to tumour cells and/or immune response and consequently influence BCG immunotherapy outcome. STn is a biomarker of concomitant molecular alterations that may further determine the tumour behaviour [140]. As such, in this study the role of STn was evaluated in the context of the response of bladder tumours to BCG immunotherapy, because nothing was known about the way patients exhibiting STn-positive tumours respond to treatment.

The study was performed in a series of 94 (38 LG, and 56 HG) NMIBC that were treated with transurethral resection followed by for BCG intravesical immunotherapy.

Approximately 66% of the studied bladder tumours were STn positive. Additionally, 10 out of 32 STn negative cases were positive for S6T, which can be considered a form of the STn antigen masked by a Gal residue *O*-3 linked to the GalNAc moiety, and so, structurally related to STn.

The expression of STn alone or in combination with S6T (STn/S6T) was associated with high-grade tumours (p=0.007; p=0.037, respectively) and also with primary tumours (p=0.001; p=0.039, respectively).

Of the 94 samples, 36 recurred after BCG treatment. The results showed that STn expression alone or in combination with that of S6T was associated with lower recurrence rates after BCG treatment. Furthermore, patients expressing STn and/or S6T presented longer recurrence free survival (RFS) and these antigens were found to be independent predictive markers of reduced recurrence after BCG immunotherapy. Moreover, recurrences after treatment displayed a reduced expression of STn antigens suggesting that BCG may be more effective against cells expressing these glycans.

STn-expressing cells presented, in the study, enhanced capability for BCG adhesion and internalisation and higher BCG-mediated cell death *in vitro*. This strongly suggests that STn expression favours BCG-mediated elimination of tumour cells, which may, in part, explain the high correlation between these glycans and treatment response. Being STn a product of incomplete proteins *O*-glycosylation, a reduction in the structural complexity of *O*-glycan may allow the *bacillus* to bind more efficiently to tumour cells [141].

On the other hand, after Clement *et al*. (2004) describe that integrin b1 chains express STn and that the antigen enhanced integrin-fibronectin adhesion, and having present that BCG is known to bind fibronectin–a5b1 integrin complexes promoting a rearrangement of cytoskeletal actin in host cells [138], which results in the phagocytosis of the pathogen [142,143], it can be thought that this might be one reason STn may contribute to a more efficient binding of the *bacillus* to tumour cells and consequently a better response to BCG.

By this time, it was a fact that STn was associated with invasion, better response with BCG immunotherapy, but still was missing the biological significance within MIBC - in Paper I only 19 MIBC were studied, while in Paper II no invasive tumours were contemplated.

Paper III confirmed that STn expression is associated with muscle invasion in a larger patient set, and provided new insights regarding its correlation with decreased survival. In this study 96 cases were evaluated – 47 classified as NMIBC and 49 as MIBC; 16 as LG and 80 as HG. STn expression was statistically

associated with histological stage ≥T1-T4 ($p<0.001$), what corroborated Paper I. When cancer specific survival (CSS) was analysed, patients with STn positive tumours had a significant lower time of life from tumour treatment ($p=0.024$). In fact, it was even possible to observe the same when the NMIBC were taken alone ($p=0.020$), and also, when T1 tumours were evaluated. The T1 tumours expressing STn had a worse CSS when compared to the T1 STn negative ones ($p<0.05$). More, STn was found to be an independent prognostic marker of worst CSS ($p<0.044$), not yet stated in any other bladder cancer paper. These results strongly reinforce the first two studies, and now with a larger series, containing both NMIBC and MIBC in equivalent proportions.

It has been demonstrated that STn expression is responsible for the modulation of cell surface glycoprotein functions in ways that favour malignant phenotypes, in different cancer models (gastric [39], breast [134]). Namely, STn expression altered the adhesive properties of cancer cells, likely by impairing integrin function, as said before [39,134]. Furthermore, it enhanced cell motility, invasion [39,134] and epithelial-to-mesenchymal transition, a key event leading to metastasis [144]. This knowledge is reinforced by the fact that in this third study, some MIBC STn positive cells were present in the front of invasion and, curiously (or not) vessels presented STn positive urothelial cells, while in STn positive NMIBC the immunostainnig was present away from the fibrovascular bundle, and no cells were observed inside the tissue vessels.

Searching for a more accurate patient stratification, the expression of phosphatidylinositol-3-kinase (PI3K)/Akt / mammalian target of rapamycin (mTOR) pathway markers in the same series of bladder tumours was also addressed.

The PI3K/Akt and the mTOR pathways are interconnected signaling cascades essential for bladder cell growth and survival. The PI3K/Akt/mTOR or mTOR pathway integrates signals initiated by nutrient intake, growth factors, and other cellular stimuli to regulate downstream signaling and protein synthesis, which ultimately leads to a competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance [145].

In the studied series the results showed that the activation of mTOR pathway proteins did not discriminate the stage of disease. Moreover it did not allow the identification of patients facing worst prognosis, which is in accordance with

previous results regarding mTOR pathway evaluation in bladder tumours [146,147].

Interestingly, advanced stage STn expressing tumours with an active PI3K/Akt/mTOR (positive pmTOR and/or pS6 - a downstream mTOR effector) showed a significantly worst CSS (p=0.027), allowing STn expression to discriminate between MIBC. More, those MIBC that presented STn positive expression concomitantly with the presence of PI3K/Akt/mTOR pathway molecules, had approximately, 6-fold risk of death by cancer (p=0.039).This was further reinforced when evaluating T2/T3 and T4 subgroups separately. Such observations strongly suggest that these particular tumours may be good candidates to mTOR-inhibitors therapeutics, such as sirolimus, an analog of rapamycin. This could be a way; however these are preliminary insights and need to be confirmed with a larger number of patients.

Adding information to these studies, STn expression as well pS6, a downstream effector of mTOR, were also evaluated in a series of ICR mice that were submitted to N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) to develop bladder cancer [148]. For the first time, it is described that chemically-induced bladder tumours express the STn antigen, therefore also mimicking the glycosylation pattern of human tumours. This is of particular importance once it opens a door to animal models studies.

The PI3K/Akt/mTOR pathway had been previously studied as well as the effect of sirolimus in the tumours of the same mice [148]. When STn and pS6 expression were evaluated, it was observed that those tumours that were treated with sirolimus had significantly lower expression in both molecules than the control groups. Despite the role played by STn in disease progression and its association with poor overall survival in humans, it can be infered that the administration of sirolimus was effective against STn positive cells. These observations also demonstrated, for the first time, an association between STn and an active PI3K/Akt/mTOR in invasive tumours.

5. CONCLUSIONS

Bladder cancer, a well studied tumour, still shows unpredictable outcomes irrespective the treatments. As so, is important to identify new biological markers or any biological mechanism that can discriminate more accurately bladder cancer outcome, adapting the treatments that will most benefit the patients.

In this work, a more comprehensive description of STn antigen expression in bladder cancer expression was achieved. In spite of the knowledge and its prognostic value in other cancers, in bladder cancer concordant information had not been yet achieved.

With the present work, it was clearly demonstrated that, STn was associated with advanced, highly proliferative tumours, invasion and organ disseminated disease, and with a significant decrease in cancer specific patient's survival. All of these findings engender STn a prognostic marker of bladder cancer.

Furthermore, STn antigen was found to be associated to high-grade NMIBC which currently constitutes one of the main therapeutics concerns due to their elevated risk of recurrence/progression. Adjuvant BCG immunotherapy has allowed to delay recurrence and decrease the risk of progression into muscle invasive disease, but still more than half of the patients either recur within two-years after TUR or show intolerance to the treatment. Taking STn expression into account it was possible to create a predictive profile of treatment response. This posttranslational modification of proteins was found to influence both the adhesion to tumour cells and immune responses and may, therefore, directly interfere with BCG action. It has been demonstrated that STn antigen correlated with a better response to BCG treatment, strongly suggesting that BCG immunotherapy is efficient against STn-positive tumours.

The present study also advocates the existence of potential more aggressive subgroup of STn positive MIBC characterized by an active mTOR-pathway, providing the first link between these two apparently unrelated events in bladder cancer (altered glycosylation and the PI3K/Akt/mTOR-pathway activation). More importantly, it sets the basis for novel therapeutic approaches against MIBC, like sirolimus, which may be translated for several other advanced stage solid tumours also known to overexpress the STn antigen.

Interestingly, it was observed that the STn antigen holds a dualism, in one hand is considered a poor prognosis factor, but on the other hand its presence benefits the treatment in patients with high grade NMIBC, but also MIBC patients. With all of the results, it is legitimated to think that vaccines towards STn, as well as the CA72-4 test to be used in serum or urine, are approaches that must be re-evaluated in the future.

A final question can be raised:

- Once a patient develops a high grade bladder cancer, would it be preferable that was a STn positive one?

6. FUTURE PERSPECTIVES

This study has provided important insights about STn prognostic value in clinical outcome of bladder cancer. Also new observations were found with respect to response to treatments. However, more studies are needed to truly disclose the role of the STn in the biology of urothelial bladder cancer.

Significant results were achived concerning STn positive expression and its association with high grade and invasive bladder tumours, and also with the overall survival. In order to better understand and reinforce these results:

- Enlarge the series is recommended, as the impact of the results will become more solid. Also ganglion metastization must be taking into account.
- It will be important to go back to the first studied series (Paper I), inserted in a prospective study, and evaluate patients' clinical outcome. It will be interesting to observe if there were recurrences and/or progression and if the positive STn primary tumours will be predictive of the different outcomes.
- It will be of large interest to analyze serum and urine samples of patients with urothelial bladder tumours of all grades and stages, with the CA72-4 test, as well as the tumours, with STn immunostaining, to understand if there is any relation that can be of clinical usefulness
- Initiate a new series of animal studies, so the preliminary results can be consolidated, is an urgent question

If the previous findings are corroborated, then a door can be open to more accurately treat bladder cancer patients, and vaccines against this glycosylated antigen can again be reconsidered.

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